The Role of Hand1 in cardiac morphogenesis

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Abstract

Hand1 encodes a bHLH transcription factor, which is essential for both placentation and cardiac morphogenesis during murine development. Extraembryonic defects in homozygous mutant embryos have precluded detailed analysis of Hand1 function in the heart. To circumvent the *Hand1*-null early lethality and gain insight into the precise function of Hand1 during vertebrate heart development the bimodal tetracycline (Tet-Off) system has been employed to generate temporally controlled loss of function and gain of function models for Hand1. The Tet-Off system comprises a tet-off transactivator and tet response element (responder) whereby transactivation of the responder and gene of interest can be switched off following addition of doxycycline. The transactivator has been targeted to the endogenous *Hand1* locus (*Tet-Off Hand1*) ensuring it is expressed in a spatial and temporal pattern consistent with the endogenous gene and generated transgenic lines for the responder (*Tre2-Hand1*). Compound heterozygotes in either *Hand1*-null or wild type backgrounds enable us to temporally switch off or over-express *Hand1* respectively.

Transactivator and responder constructs for gene targeting and transgenics respectively have been tested extensively *in vitro*, and sent to a commercial company to generate the mouse strains. Germline transmission was achieved for both *Tet-Off-Hand1* and two transgenic *Tre2-Hand1* strains. The *Tre2-Hand1* responder lines failed to express *Hand1* at a level suitable to rescue the mutant phenotype when crossed with the *Tet-Off-Hand1* driver on a *Hand1*-null background; thus preventing further manipulation via dox administration to pregnant mothers to turn off *Hand1* at different embryonic stages. However, a third line suitably expressed *Hand1* under the tet-system and is currently being crossed with the driver for determining rescue as a first step towards validating the loss of function model.

The transactivator and responder lines have also been crossed to generate mice that over-express *Hand1*. At E9.5, embryos that over-express *Hand1* have an extended outflow tract, convoluted looping of the linear heart tube and a small, thick-walled ventricle, which lacks a defined lumen. Marker analysis reveals inappropriate cardiomyocyte differentiation and an over-proliferation in the affected areas with a lack of ballooning in the presumptive left ventricle. *In vitro* differentiation studies have also been performed in ES cell lines that stably and precociously over-express *Hand1* to complement the *in vivo* model.

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Glossary of terms and abbreviations

129Sv	a mouse strain
AD	activation domain
AHF	anterior heart field
ANF	atrial natriuretic factor
AV	atrioventricular
AVC	atrioventricular canal
β-Gal	beta-galactosidase
bHLH	basic helix-loop-helix
BMP	bone morphogenetic protein
bp	base pair
BSA	bovine serum albumin
cDNA	complimentary deoxyribonucleic acid
CHD	congenital heart disease
CMV	cytomegalovirus
CN	copy number
cps	counts per second
CS	calf serum
D12	ES cell line over-expressing *Hand1*
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOC	sodium deoxycholate
Dox	doxycycline
DPX	dibutyl phthalate xylene
DTT	
E	embryonic stage (mouse)
EB	embryoid body
EDTA	ethylene diamine tetra-acetate
EGFP	Enhanced Green Fluorescent protein

EMT	epithelial to mesenchymal transformation
ES cells	embryonic stem cells
FBS	foetal bovine serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
Hand1	Heart and neural crest derivatives expressed transcript 1
HEK 293	human embryonic kidney cell line
HRP	horseradish peroxidase
Hyg	hygromycin
IF	immunofluoresence
ISH	*in situ* hybridisation
kb	kilo-base
kDa	kilo-Dalton
LIF	Leukaemia inhibitory factor
MEMα	Minimum Essential medium α
Mlc2v	myosin light chain 2 ventricular isoform
mRNA	messenger ribonucleic acid
NIMR	National Institute for Medical Research
NP-40	nonidet P-40
OFT	outflow tract
OPT	optical projection tomography
PBS	phosphate buffered saline
pCAGtTA	Construct containing Tet-Off transactivator under the control of the chick β-actin promoter
PCR	polymerase chain reaction
PGKneo^r	neomycin resistance cassette under the control of the PGK promoter
Puro	puromycin
R1	wildtype ES cell line
RA	retinoic acid
RDA	representational differential analysis
RLU	relative light units

RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcriptase PCR
rtTA	Tet-On transactivator
SA	sinoatrial
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrlyamide gel electrophoresis
Tc	tetracycline
TEMED	N, N, N', N'-tetramethyl-ethylene diamine
TESPA	3-aminopropyl-triethoxysilane
Tet-Hand1	tet-responsive *Hand1* transgenic strain from collaborators (T.Mohun, NIMR)
tetO	tet operator sequences; Tre2 consists of seven consecutive tetO repeats
Tet-Off-Hand1	Tet-Off transactivator targeting construct
TG39	wildtype ES cell line that carries the silent *Tre2-Hand1* transgene
TGFβ	transforming growth factor beta
TK promoter	thymidine kinase promoter
TRE	Tet response element
Tre2	Tet response element 2 (modified TRE)
Tre2-Hand1	tet-responsive *Hand1* transgene
Tre2-Hand1-EGFP	tet-responsive *Hand1* targeting construct
TRITC	tetramethylrhodamine isothiocyanate
TS	cells trophoblast stem cells
tTA	Tet-Off transactivator
tTS	transcriptional silencer for the Tet-On system
UV	ultraviolet
X-Gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
Amps	ammonium persulphate
DTT	dithiothreitol
SOPF	specific and opportunistic pathogen free
SPF	specific pathogen free

Declaration

I, Catherine Risebro, hereby declare that, unless otherwise stated, the experimental data contained within this thesis submitted for the Degree of Doctor of Philosophy is my own work.

Acknowledgements

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Chapter 1: General Introduction

1.1 Heart development

1.1.1 Introduction

The heart is the first organ to form and function during embryogenesis in vertebrates, beginning soon after gastrulation. In the mouse this occurs at around embryonic day 7 (E7) in mice (Figure 1.1). First, the cardiac precursors are specified in regions of anterior lateral plate mesoderm either side of the embryonic midline, known as the cardiac crescent. The precursors migrate medially from the cardiac crescent to form the linear heart tube. The linear heart tube initiates rhythmic contractions and undergoes rightward looping, after which the chambers are specified, septate and mature into the four-chambered organ.

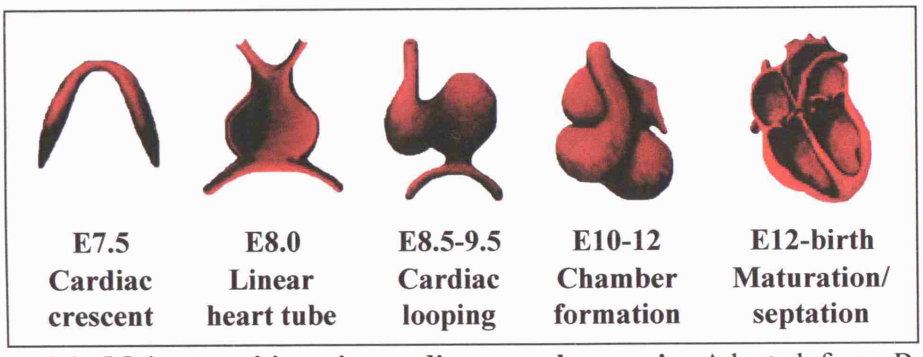
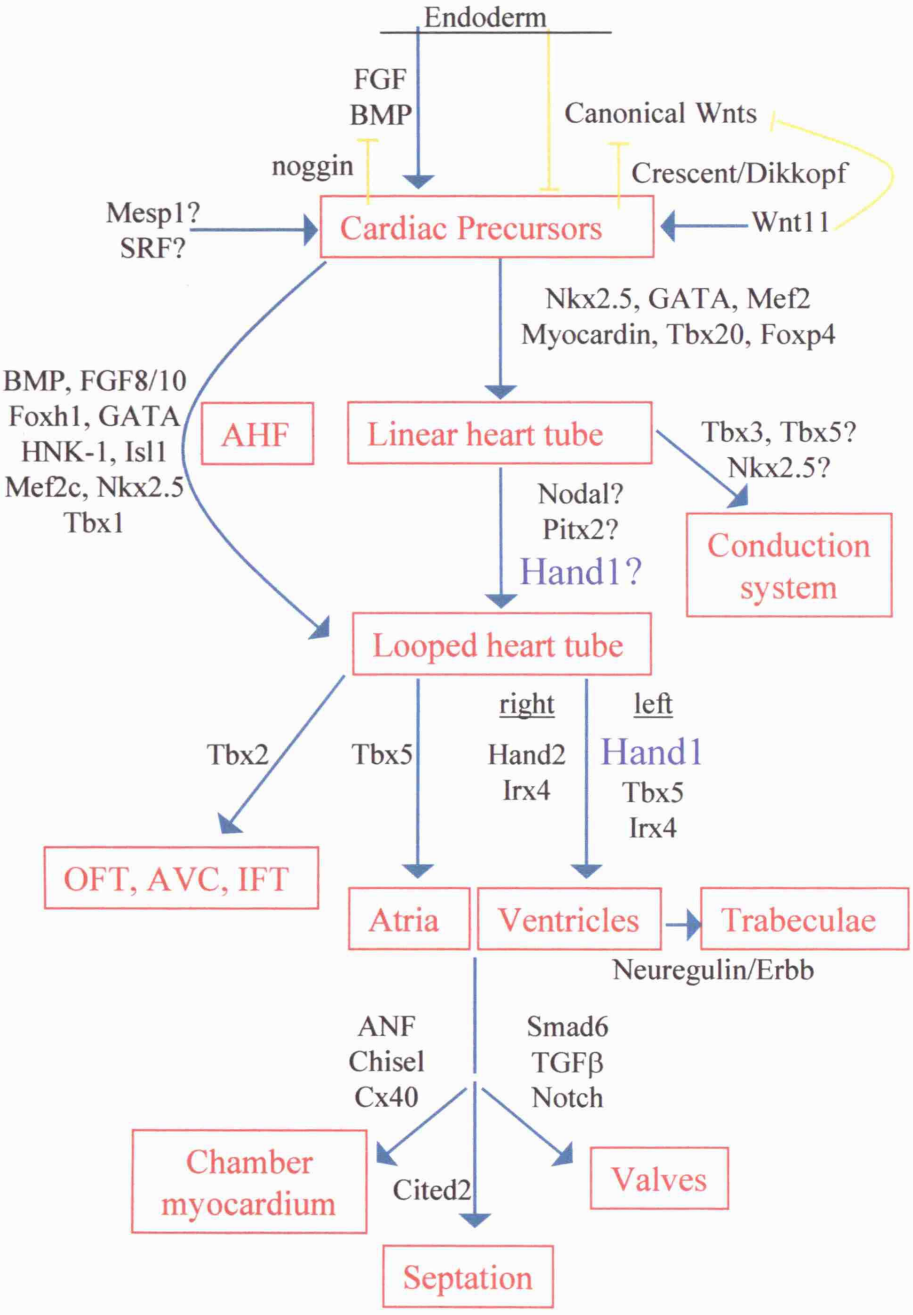


Figure 1.1. Main transitions in cardiac morphogenesis. Adapted from Bruneau, 2002. Cardiac morphogenesis can be artificially broken down into stages, the main stages are depicted in these cartoons. Heart development begins at E7.5 with formation the cardiac crescent, which fuses at the embryonic midline to form the linear heart tube at E8. Cardiac looping and the initiation of chamber morphogenesis then take place between E8.5 to E9.5, followed by chamber formation between E10 and E12. From E12 until birth, the chambers of the heart undergo maturation, septation and valve formation.

Cardiac morphogenesis involves many complex transcriptional networks (Figure 1.2), which rely on associations between both lineage specific and more widely expressed

Figure 1.2. Transcription factor involvement in cardiac morphogenesis. Overview of signalling pathways and transcription factors involved, or suspected to be involved throughout cardiac morphogenesis, from the specification of precursors through to the formation of the mature four-chambered organ.


Endoderm
FGF
BMP
Canonical Wnts
noggin
Crescent/Dikkopf
Mesp1?
SRF?
Cardiac Precursors
Wnt11
Nkx2.5, GATA, Mef2
Myocardin, Tbx20, Foxp4
BMP, FGF8/10
Foxh1, GATA
HNK-1, Isl1
Mef2c, Nkx2.5
Tbx1
AHF
Linear heart tube
Tbx3, Tbx5?
Nkx2.5?
Nodal?
Pitx2?
Hand1?
Conduction system
Looped heart tube
right
left
Tbx2
Tbx5
Hand2
Irx4
Hand1
Tbx5
Irx4
OFT, AVC, IFT
Atria
Ventricles
Trabeculae
Neuregulin/Erbb
ANF
Chisel
Cx40
Smad6
TGFβ
Notch
Chamber myocardium
Cited2
Valves
Septation

transcription factors. The transcription factors involved come from a diverse range of transcription factor families, including basic helix-loop-helix (bHLH), homeobox, T-box, zinc finger and MADS domain. Many cardiac transcription factors have been shown to interact with each other to activate cardiac genes and induce cardiomyocyte differentiation, such as GATA4 And Nkx2.5 (Durocher et al., 1997), Nkx2.5 and Tbx5 (Hiroi et al., 2001). Deletion of these early cardiac transcription factors generally results in arrested cardiac development. A further level of complexity is added to the cardiac transcription network by co-activators. GATA4 is regulated positively and negatively by FOG2 (Friend-of GATA 2)(Lu et al., 1999; Svensson et al., 2000), and is positively regulated by co-activator p300 (Dai and Markham, 2001). Transcriptional co-activator activity is also controlled, CITED (CBP/p300-interacting transactivator with ED-rich tail) factors, for example, have been shown to bind to and regulate p300 (Bhattacharya et al., 1999), and CITED2 has been shown to interact with TFAP2 (transcription factor AP2)(Bamforth et al., 2004). As a result of the complex network of transcription factors involved in cardiogenesis, many cardiac specific genes are regulated by multiple enhancers, which direct expression to both overlapping and distinct regions, within the developing heart.

Transcription factors involved in early cardiac morphogenesis are conserved between species while pathways specific to vertebrates regulate later stages of vertebrate heart development (from cardiac looping onwards). Within these transcriptional networks, control of cardiac gene expression has been found to be modular requiring multiple cis-regulatory modules (Cripps and Olson, 2002). Perturbations in the molecular or morphogenetic pathways can result in early foetal mortality due to heart failure or at best congenital heart disease (CHD).

1.1.2 Congenital heart disease

Congenital heart disease (CHD) occurs in humans in 8 of 1000 live births (Hoffman, 1995) and as such is the leading cause of congenital childhood death. Additionally, congenital cardiac anomalies account for 10% of all spontaneous abortions. CHDs tend to affect segments of the heart, rather than the whole heart, reflecting the modular fashion of heart development (Fishman and Olson, 1997). One example is in the cases of hypoplastic left or right ventricle, where the unaffected ventricle is morphologically and physiologically normal. Around 30% of congenital heart defects manifest as

outflow tract abnormalities (Kelly and Buckingham, 2002) where the great vessels (aorta and pulmonary artery) do not make appropriate contacts with specific chambers, resulting in phenotypes such as double outlet right ventricle (DORV) and truncus arteriosus (TA).

Cardiac transcription factors play a crucial, fundamental role in heart development, and are conserved across species. Studying the function of such transcription factors to understand the 'normal' developmental situation in the heart at the molecular level is of wide interest, and is particularly relevant in extrapolating to the morphogenetic anomalies of the disease situation.

1.1.3 Cardiac morphogenesis

Development of the vertebrate heart can be artificially broken down into distinct stages (Figure 1.1; (Fishman and Chien, 1997; Fishman and Olson, 1997)). The following discusses each of these stages primarily in the developing mouse heart, with specified reference throughout to other model organisms including chick, zebrafish and *Xenopus*.

1.1.3.1 Specification of the cardiac precursors

Fate-mapping experiments identified a bilateral splanchnic mesodermal population of cells in the anterior lateral mesoderm that subsequently contribute to the early heart tube (Srivastava and Olson, 2000; Chen and Fishman, 2000). As the embryo folds ventrally, these paired cardiac precursors migrate medially and fuse in the ventral, anterior region of the embryo (adjacent to the head folds), forming the cardiac crescent at E7.5 (Figure 1.3). The first cardiomyocytes begin to differentiate from the cardiac crescent. The inducing signals for cardiomyocyte differentiation arise from the underlying endoderm in the form of bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs) (Figure 1.2) ((Chen and Fishman, 2000); (Lough and Sugi, 2000); (Cripps and Olson, 2002).

In addition, the boundaries of the cardiac crescent are defined by repressive signals from members of the Wnt family of growth factors that signal through the canonical (β-catenin) pathway. Within the cardiac crescent, Wnt antagonists Crescent and/or Dickkopf-1 are secreted, blocking the repression and committing cells to a cardiac fate (Cripps and Olson, 2002). In addition to Wnt inhibitors, the BMP inhibitor Noggin has

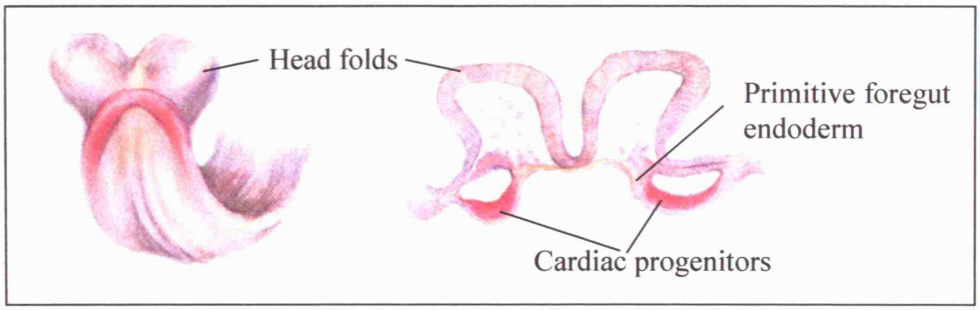


Figure 1.3. The cardiac crescent. (Adapted from Harvey (2002). (Left) Ventral view of a whole mouse embryo at E7.5 and (right) a representative transverse section. Cardiac precursors are shown in red. The cardiac precursors are first recognisable as the cardiac crescent in the anterior, ventral region of the developing embryo bordering with the head folds. Cardiogenic inducing signals are received from the underlying endoderm.

been implicated in cardiomyocyte induction. *Noggin* is expressed strongly in the heart-forming regions between E7.0 and E8.0 and treatment of undifferentiated ES cells with Noggin results in a marked increase in cardiomyocyte differentiation (Yuasa et al., 2005). Non-canonical Wnt signalling by Wnt11, through the JNK pathway and possibly by inhibiting signalling through the canonical pathway, in the anterior lateral mesoderm has also been shown to be crucial for cardiomyocyte induction in *Xenopus* (Pandur et al., 2002), and further that it promotes cardiomyocyte formation in mouse embryonic carcinoma (P19) cells (Pandur et al., 2002) and in mouse ES cells (Terami et al., 2004). It has also been suggested that Wnt11 signalling may serve as an integrator between Wnt and BMP signalling (van den Hoff et al., 2004) as JNK-mediated BMP signalling was found to be involved in cardiomyocyte formation in addition to Smad-mediated BMP signalling (Monzen et al., 2002).

In response to BMP induction and antagonism of canonical Wnt signalling, many cardiac transcription factors are induced in the cardiac crescent (Figure 1.2). A notable example is the homeobox transcription factor Nkx2.5, which is the earliest known marker for the cardiomyogenic lineage (Komuro and Izumo, 1993; Lints et al., 1993). Other cardiac transcription factors induced include Mef2 (myocyte enhancer factor), GATA factors, the Hand genes Hand1 and Hand2, Irx 4 (Bao et al., 1999; Bruneau et al., 2000) and Tbx5/20 (Harvey, 2002). GATA factors (GATA4, 5 and 6) co-operate with Nkx2.5 to induce activation of cardiac specific genes, and also regulate each other's expression through mutually reinforcing positive feedback loops (Srivastava and

Olson, 2000). It seems that much of the cardiac pattern is laid down at this cardiac crescent stage. Evidence for this arises from experiments involving the addition of the morphogen retinoic acid where an effect of exogenous retinoids on the heart was only observed if applied at the crescent stage (Xavier-Neto et al., 1999).

1.1.3.2 Migration of precursors to form the linear heart tube

At around E8.0, the cardiac crescent fuses from the anterior to the posterior end, forming a primitive linear heart tube in the shape of an inverted 'Y' along the ventral midline of the embryo, and is attached to the body wall by the dorsal mesocardium (Figure 1.4). This migration depends on a gradient of fibronectin along the A/P axis deposited in the extracellular matrix between the mesoderm and endoderm (Harvey, 2002). Additionally, GATA factors have been implicated in the formation of the linear heart tube (Figure 1.2), for example, in *GATA4*-null embryos the precursors remained bilateral, failing to migrate to the midline and form the linear heart tube, and subsequently fail to undergo cardiac morphogenesis ((Kuo et al., 1997); (Molkentin et al., 1997). Studies such as these have led to the belief that fusion of the bilateral cardiac pimordia at the ventral midline is absolutely essential for cardiac morphogenesis (Srivastava and Olson, 2000; Brand, 2003). Recently however, it has been shown that the later stages of cardiac morphogenesis, establishing chamber identity, left-right

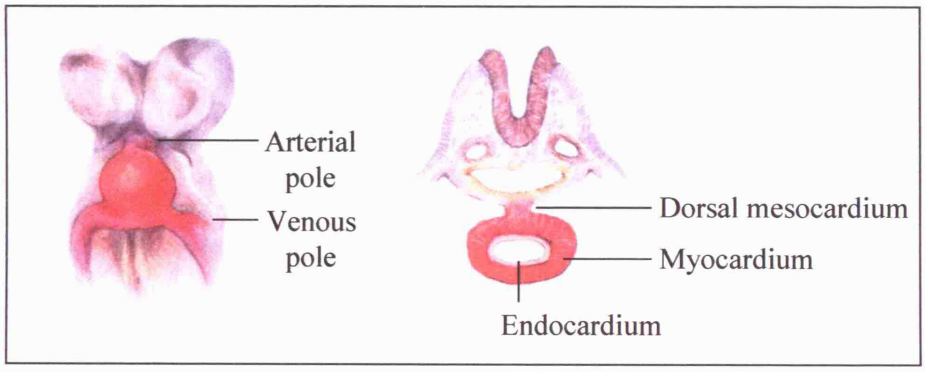


Figure 1.4. The linear heart tube. (Adapted from Harvey (2002). (Left) Ventral view of a whole mouse embryo at E8.25, and (right) a representative transverse section. Myocardium is shown in red. The cardiac progenitors migrate medially and ventrally and fuse to form the linear heart tube. The linear heart tube consists of an outer tube of myocardium with an endocardial lining, and has separate venous (inflow) and arterial (outflow) regions. The heart tube remains attached to the embryo via the dorsal mesocardium.

asymmetry and looping morphogenesis, do not require heart tube fusion (Li et al., 2004). In *Foxp4*-null embryos midline fusion does not occur, the embryos instead develop two bilateral, complete hearts in which chamber specification and septation is correct, cardiac left-right asymmetry is properly specified and looping occurs appropriately (Li et al., 2004). Furthermore, this study indicates that the cardiac mesodermal precursors are programmed to a high degree and possess all relevant information necessary for heart development.

The linear heart tube consists of an outer myocardium around a tube of endocardial cells, separated by cardiac jelly (an extracellular matrix) (Figure 1.4). The linear heart tube spontaneously initiates contraction at around E8.5 from its posterior end. The first contractions are peristaltic, but soon become sequential, with atrial contraction preceding that of the presumptive common ventricle (Fishman and Chien, 1997).

The linear heart tube has separate inflow (venous) and outflow (arterial) regions. Initially, cell fate analyses indicated that the linear heart tube was patterned along its anterior to posterior (A/P) axis into segments that correspond to the future aortic sac, outflow tract, right and left ventricle, and atria (Yutzey and Bader, 1995). However, with the discovery of the anterior heart field, a second population of cardiac precursors, the linear heart tube has been shown to give rise mainly to the left ventricle (Kelly and Buckingham, 2002; Cai et al., 2003; Zaffran et al., 2004).

1.1.3.3 A second cardiac lineage

1.1.3.3.1 The anterior heart field

Studies in chick and mouse identified a population of myocardial precursor cells in the pharyngeal mesoderm, anterior to the early heart tube, which has been termed the anterior, or secondary, heart forming field (Kelly and Buckingham, 2002). The anterior heart field (AHF) is specified in the splanchnic mesoderm, at cardiac crescent stage with the other heart precursors, located medial and dorsal to the classical cardiac crescent (Kelly et al., 2001; Cai et al., 2003) (Figure 1.5). As the linear heart tube is formed, the anterior heart field becomes located in a more dorsal and anterior position. The anterior heart field was shown to generate the outflow tract myocardium, a region added after ventricle and inflow tract formation (Kelly et al., 2001) and contributes significantly to the myocardium of the right ventricle in mice between E8.25 and E10.5, at the point of

cardiac looping (Zaffran et al., 2004). DiI/rhodamine labelling of anterior heart field cells in the chick has since identified two temporally distinct populations of cells moving into the arterial pole of the heart tube (Waldo et al., 2005b). The first group of cells migrated into the distal outflow tract in a spiral manner (cells originating on the right side of the anterior heart field end up on the left side of the OFT, and vice versa) and differentiated into myocardium. Once elongation of the outflow tract myocardium is complete, a second group of cells migrates vertically from the anterior heart field into the aortic sac that have been designated vascular smooth muscle progenitors. After septation of the aortic sac, these cells differentiate into true vascular smooth muscle. Therefore, in chick, in addition to contributing myocardium, the anterior heart field also adds vascular smooth muscle to the arterial pole of the developing heart, but this remains to be determined in the mouse (Waldo et al., 2005b).

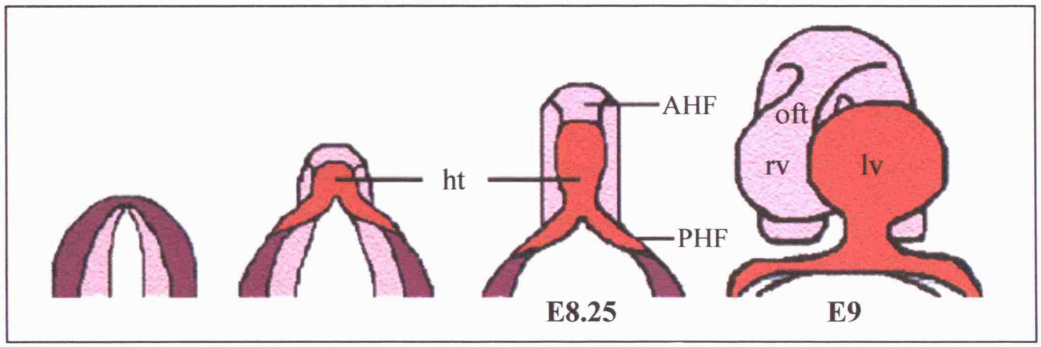


Figure 1.5. The anterior heart field. (Adapted from Kelly et al., 2001). Cartoon illustrating the anterior heart field (pink) in relation to the classical heart-forming region (purple), the cardiac crescent, and to the linear heart tube (red) during early heart formation, as marked by expression of the *Fgf10* enhancer trap-like LacZ transgene. The anterior heart field lies medially to the cardiac crescent, and later becomes positioned dorsal and anterior to the linear heart tube. During cardiac looping, the anterior heart field gives rise to the right ventricle and the outflow tract regions of the developing heart. AHF, anterior heart field; ht, heart tube; lv, left ventricle; oft, outflow tract; PHF, primary heart field; rv, right ventricle.

1.1.3.3.2 Transcriptional control in the anterior heart field

The presence of the anterior heart field suggests that the heart is formed from two distinct myocardial precursor populations that appear to have independent genetic programmes. The same families of transcription factors seem to be involved up to a point, although the signals originate from different sources (Waldo et al., 2001) and

separate enhancers exist to drive expression within the anterior heart field to those that drive expression in the classical heart field. One such enhancer has been identified for *Mef2c*, that drives expression of *Mef2c* in the precardiac mesoderm and subsequently in the anterior heart field and its derivatives the outflow tract and left ventricle, but not to other regions of *Mef2c* expression (Dodou et al., 2004).

Fgf signalling appears to have a role in migration of anterior heart field precursors and their differentiation (Kelly et al., 2001; Waldo et al., 2001). *Fgf10* is expressed in the cardiac precursors in the pharyngeal mesoderm. Kelly et al. (2001) generated an enhancer trap-like *nlacZ* transgene inserted upstream of the *Fgf10* gene that is under transcriptional control of the *Fgf10* gene regulatory sequences. LacZ positive cells were seen to move from the pharyngeal mesoderm to the distal outflow tract indicating a role for Fgf10 in the migration of precursors to the developing heart. There is also evidence for BMP influence in recruitment and differentiation of cells of the secondary heart field (Waldo et al., 2001; Mjaatvedt et al., 2001). *BMP2* is expressed in the anterior heart field and outflow tract myocardium and appears to induce differentiation of cells to myocardial lineage, as treatment of anterior heart field explants with BMP-inhibitor noggin promoted proliferation and impaired myocardial differentiation (Waldo et al., 2001). Additionally, Fgf8 may also play a role in co-ordination of outflow tract morphogenesis as a BMP antagonist, preventing premature differentiation (Waldo et al., 2001). *Nkx2.5* and *Gata4* are both expressed in the cells of the anterior heart field prior to their differentiation (Waldo et al., 2001), but their expression depends on alternative cis-acting regulatory elements to those utilised in the rest of the heart (Schwartz and Olson, 1999). The *Nkx2.5/Gata4* expressing cells also begin to express *HNK-1* then translocate from the anterior heart field to the outflow tract (Waldo et al., 2001). HNK-1 is involved in cell-cell or cell-substrate interactions, normally associated with migratory populations, particularly neural crest cells. These cells only begin to differentiate into cardiomyocytes as they approach and integrate into the existing developing heart (Waldo et al., 2001).

There are also differences in regulation between the anterior and primary heart fields. Several transcription factors have been identified that impact solely upon the anterior heart field and it's derivatives. The forkhead transcription factor family has recently come to the fore, with many of its members being implicated in cardiac development. One such example is Foxh1 which appears to be essential for the development of the

anterior heart field and consequently the OFT and RV (Von Both et al.,2004·). Accordingly, *Foxh1*-null embryos form primitive heart tube but do not form OFT and RV. Interestingly, *Foxh1*-null embryos do not express outer curvature markers of future working myocardium suggesting that Foxh1 is also involved in differentiation of the cardiac chambers (Von Both et al., 2004). *Mef2c* is a direct target for Foxh1. Von Both and co-workers (2004) showed that Foxh1 interacts with Nkx2.5 to induce Smad-dependent activation of a *TGF-β* response element in the *Mef2c* promoter region.

The T-box (Tbx) family of transcription factors are highly conserved and are critical for the development of a wide variety of organs (Kiefer, 2004). *Tbx1* is a T-box transcription factor that is expressed in anterior heart field progenitors, downstream of Nkx2.5, where it regulates, but is not required for, the addition of myocardium to the outflow tract in a non cell-autonomous manner. Loss of Tbx1 in this region results in slightly decreased levels of proliferation in the anterior heart field and in fewer cardiomyocytes being added to the OFT most likely due to the reduction in precursors (Xu et al., 2004).

The LIM homeodomain transcription factor Isl1, has also been shown to be critical for proliferation and survival of the anterior heart field, and additionally for the migration of these precursors into the heart tube (Cai et al., 2003). *Isl1* progenitors exist in anterior and posterior splanchnic mesenchyme and migrate into the heart at both the anterior and posterior ends, when the splanchnic mesenchyme is contiguous with the myocardium. *Isl1* expressing cells, therefore, contribute the majority of cells to the outflow tract, right ventricle and atria, and also to specific regions of the left ventricle. Consequently, *Isl1*-null embryos die at around E10.5 and lack outflow tract and right ventricle (Cai et al., 2003). This suggests Isl1 plays the major role in cardiomyocyte specification in the second population of cardiac precursors, a function assigned to Nkx2.5 in the primary heart field. Supporting this, Isl1 and GATA4 have been shown to direct *Mef2c* expression in the anterior heart field, the implication being that GATA factors and Isl1 are the key regulators in the anterior heart field (Dodou et al., 2004). Another T-box transcription factor, Tbx20, has also been identified as a partner for Isl1, controlling expression of key transcription factors via anterior heart field specific enhancers (Takeuchi et al., 2005).

It has been postulated that Isl1 is required to maintain the undifferentiated state of the anterior heart field progenitors. *Isl1* expressing cells are undifferentiated and as they

enter the myocardium *Isl1* becomes down-regulated coincident with differentiation (Cai et al., 2003). Furthermore, given that the *Isl1* precursors contribute to more regions of the developing heart, specifically at both anterior and posterior ends of the heart tube, the previously described anterior heart field could be a subset of a much larger pool of progenitors (Cai et al., 2003), which has since been collectively defined as the 'second lineage' (Meilhac et al., 2004).

1.1.3.3.3 Single common progenitor for the cardiac lineage

The studies on Isl1 have identified a large pool of cardiac progenitors of which it was hinted that the AHF was a smaller sub-population. Since then it has been uncovered that the Isl1 progenitors may also be a subset of the pool of cardiac progenitors.

Retrospective clonal analysis of myocardial cells has confirmed the existence of two separate cardiac lineages, but revealed that each contributes slightly differently to the developing heart than the previously defined primary and anterior heart fields (Meilhac et al., 2004). The studies indicated that the left ventricle was derived entirely from the first lineage and the outflow tract was derived entirely from the second lineage, yet both lineages were shown to contribute to all other regions of the heart (Meilhac et al., 2004).

The retrospective clonal analysis also provided evidence for a single common heart progenitor population that is specified around gastrulation (E6.5)(Figure 1.6; (Meilhac et al., 2004). It has, therefore, been postulated that the two heart fields may actually be classed as a single heart-forming region (van den Hoff et al., 2004). When the embryo involutes, the cardiogenic mesoderm located medially to the 'classic' heart field is displaced laterally, and upon heart tube formation, this region of mesoderm is displaced dorsal to the heart tube and ventral to the pharynx (Kelly and Buckingham, 2002), but remains contiguous with the heart tube via the inflow and outflow tracts. Due to positional differences each region receives different cues resulting in the anterior portion maturing at a later stage (van den Hoff et al., 2004) and appearing to be a separate entity, and has thus been termed the second lineage.

In support of this possibility, the bHLH transcription factor Mesp1 is expressed in the cardiogenic mesoderm at gastrulation and as such is thought to be the earliest indicator of cardiovascular fate (Saga et al., 2000). Retroviral labelling studies previously indicated that endocardium and myocardium shared very little lineage relationship

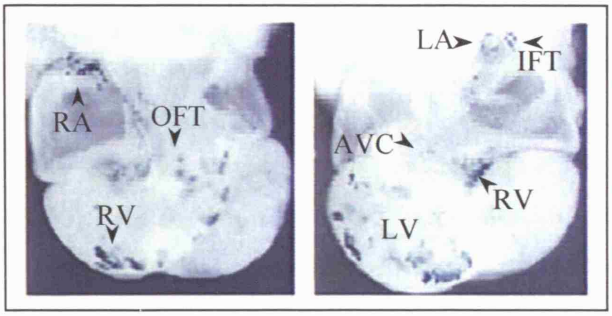


Figure 1.6. A single common cardiac progenitor. (Adapted from Meilhac et al., 2004) E10.5 heart viewed from ventral (left) and dorsal (right) aspects. Retrospective clonal analysis using the *laacZ* reporter gene targeted to the *α-cardiac actin* locus demonstrates the existence of a common cardiac progenitor. LacZ positive staining cells are identified in all regions of the developing heart derived from both the primary heart field and the second lineage. RA, right atrium; RV, right ventricle; OFT, outflow tract; AVC, atrioventricular canal; LV, left ventricle; LA, left atrium; IFT, inflow tract.

(Mikawa et al., 1992). However, lineage tracing studies have shown that *Mesp1* positive mesoderm is pluripotent and contains the precursors for the entire heart, including all of the endocardial/endothelial, epicardial and myocardial lineages (Saga et al., 2000). Furthermore, the ascidian *Mesp* gene has been shown to be essential for the development of heart progenitors and consequently the heart, and for the expression of cardiac transcription factors such as *Nkx* and *HAND* (Satou et al., 2004). It is possible, therefore, that the vertebrate *Mesp* genes are also involved in specifying cardiac precursors, however, a precise role for Mesp1 in mouse heart development remains to be resolved.

1.1.3.4 Rightward (dextral) looping of the linear heart tube

Cardiac looping is a process that is conserved across all vertebrate species, reflecting its critical importance in heart development. As yet, the precise cellular and molecular mechanisms underlying the looping process are unknown. Looping is the first morphological manifestation of left-right asymmetry in the developing embryo, converting the anterior-posterior pattern of the heart into a left-right pattern. Cardiac looping ensures that the presumptive chambers are in the correct orientation and alignment with respect to the inflow and outflow regions, which will ultimately become the great vessels of the heart.

The linear heart tube loops rightwards between E8.0 and E8.5 forming a structure with a common atrium and a common ventricle (Figure 1.7) that is properly aligned for future integration into the vascular network. First, there is a 'jog' or displacement of the heart tube to the right (also called c-looping), subsequently followed by rightward (or dextral) looping accompanied by a rotation of the heart tube around an anterior/posterior axis (s-looping) (Manner, 2000). As the heart tube loops, the atrial myocardium and future venous tributaries are displaced anteriorally and dorsally with respect to the ventricles. The heart tube increases in size four to five fold during looping (Kelly and Buckingham, 2002), largely attributed to contributions from the second cardiac lineage, including the anterior heart field.

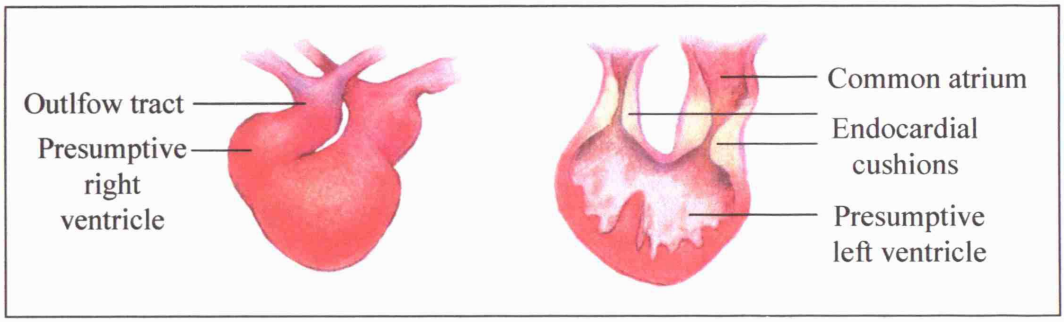


Figure 1.7. The looped heart. (Adapted from Harvey (2002). (Left) Isolated mouse heart at E10.5, and (right) representative frontal section. Myocardium is shown in red. The linear heart tube undergoes rightward cardiac looping, forming a structure with a common atrium and a common ventricle. During looping, the common atria is displaced anteriorally and dorsally so that it is above the ventricles. Looping ensures the chambers are correctly aligned for integration into the vascular network. Endocardial cushions are also forming in the outflow tract and atrioventricular canal, which are the precursors of the valves and septae of the heart.

The mechanism of cardiac looping is unknown, but studies have suggested that changes in cell shape, cell-cell interaction and/or cell migration occur coincidentally with cardiac looping. Recently, it has been demonstrated that active actin polymerisation on both sides of the heart tube is absolutely required to drive myocardial cell shape changes to bring about c-looping (Latacha et al., 2005). In terms of controlling cardiac looping directionality, several extracellular matrix molecules are asymmetrically expressed in the precardiac mesoderm, and it has been suggested, therefore, that looping may be regulated by alterations of the extracellular matrix (ECM) resulting in cardiomyocyte migrations in the linear heart tube that correlate with the direction of looping (Smith et

al., 1997; Tsuda et al., 1996). In support of this theory, chick embryos treated with an antibody against the ECM molecule flectin display randomised heart looping either to the right or left, or not at all (Linask et al., 2002). In the chick embryo, *flectin* is expressed in the left side of the precardiac mesoderm in the lateral plate. Furthermore, *flectin* is expressed asymmetrically, predominantly left-sided, in the dorsal mesocardium and in the splanchnic mesoderm that correlates to the anterior heart field, prior to and during looping (Linask et al., 2003). The myocardium that expresses *flectin* is suggested to be more compliant and 'less stiff', allowing it to stretch and balloon out forming the outer curvature of the heart (Linask, 2003). However, it has been shown that it is the asymmetrical expression of *flectin* within the dorsal mesocardium, not the myocardium or the cardiac jelly, that is associated with delineating the direction of cardiac looping (Linask et al., 2003). The expression of *flectin* is slightly different in the looping mouse heart, but it is expressed asymmetrically with a right-sided predominance in the outflow tract and left-sided in the left ventricle (Tsuda et al., 1998). Despite the differing expression patterns in chick and mouse, *flectin* expression seems related to looping and it may also be involved in the instigation of cardiac looping.

Asymmetric cell proliferation in the dorsal mesocardium and the splanchinic mesoderm of the anterior heart field has also been linked to cardiac looping, and may be controlled in part by matrix metalloproteinases (MMPs), in particular MMP-2 (Linask et al., 2005). During rightward looping, there are an increased number of proliferating cells in the left dorsal mesocardium, which are assumed to drive looping to the right although the precise mechanism is still under investigation.

Following descriptive studies that suggested the heart rotates around the dorsal mesocardium, a simulation model of the looping chick heart was constructed (Manner, 2004). The model indicated that the direction of heart looping could be controlled by rotation of the arterial pole, in the case of normal dextro-looping the arterial pole rotates to the right. Then during the subsequent phases of cardiac looping the venous pole rotates to the left, complementing the initial rightward rotation. It was therefore suggested that molecules displaying asymmetrical left-right expression in the cranial and caudal poles might provide insight into the directionality of heart looping (Manner, 2004).

In terms of insight into the upstream molecular mechanisms underlying looping, differential expression of genes along the inner and outer curvature of the heart tube

may be important, for example the left sided expression of *Pitx2*. *Pitx2* is expressed on the left side of developing organs and interprets the asymmetric left-right signals from Sonic Hedgehog/Nodal (Srivastava and Olson, 2000). *Pitx2* overexpression in the right lateral plate mesoderm in chick embryos results in symmetrical unlooped hearts, indicating a possible role in looping for Pitx2 (Logan et al., 1998). Yet in *Pitx2* knockout mice, the heart loops normally but loses its left-sided identity (Kitamura et al., 1999). Similarly, mice lacking only the Pitx2c isoform (the Pitx2 isoform that is specifically left-determining) display laterality defects, but not of looping or within the ventricles (Bamforth et al., 2004). Furthermore, in *Foxp4*-null embryos where midline fusion of the cardiac primorida does not occur, and embryos develop two bilateral, appropriately specified complete hearts, *Pitx2* is expressed throughout the left-sided heart, but is absent from the right-sided heart (Li et al., 2004). The mechanism and molecular basis of looping is more complex, therefore, than the interpretation of left-right axis formation. Nodal is also a potential candidate for control of looping morphogenesis (Brand, 2003). Ectopic expression of *nodal* on the right side of chick embryos affects cardiac looping (Levin et al., 1995) and in the mouse, a hypomorphic allele of *nodal*, amongst other defects results in randomised direction of heart looping (Lowe et al., 2001).

Shear forces of blood-flow also probably contribute to normal looping of the heart (Hove et al., 2003). Beads placed in zebrafish embryo hearts at 37 h.p.f (hours post fertilisation) that blocked blood flow both in and out of the primitive heart, caused a failure of looping, a lack of bulbus formation and collapse of the inflow and outflow tract walls. The most likely cause of this phenotype was decreased shear forces arising from decreased blood flow (Hove et al., 2003). In addition, the external forces of surface tension have been shown to have an effect on cardiac looping in chick embryos in culture (Voronov and Taber, 2002; Voronov et al., 2004). These experiments imply a possible function of the force imposed by the extraembryonic splanchnopleura membrane on the ventral side of the embryo, guiding correct direction of looping.

Correct haemodynamics is known to be important for myocardial growth, structure and function in the adult heart (Rockman et al., 1994), and it is also thought to be crucial for the developing embryonic heart as experimentally reduced load delayed growth and morphogenesis of the ventricles (Sedmera et al., 1998). In *Mlc2a*-null embryos, in which the atria do not have proper myofibrillar organisation and consequently beat at

severely diminished rates, there are secondary abnormalities in cardiac chamber morphology and looping morphogenesis that were attributed to altered haemodyanmics due to loss of atrial function (Huang et al., 2003).

1.1.3.5 Remodelling of the heart - Chamber maturation, septation, endocardial cushion formation

After looping, the chambers become distinct as the heart tube septates into a four-chambered structure in which the systemic and pulmonary circuits are distinct, and the valves develop (Figure 1.8). Each chamber differs in pattern of gene expression. Again, transcription factors have been implicated in chamber specification, most notably those with chamber specific expression patterns (Figure 1.2), for example Hand1 and Hand2 in the left and right ventricles respectively (Srivastava et al., 1997). The Iroquois homeobox transcription factor *Irx4* is expressed only in the ventricular chambers (Bao et al., 1999; Bruneau et al., 2000)), while *Tbx5* is expressed in the posterior portion of the developing heart, the inflow tract and left ventricle (Bruneau et al., 1999).

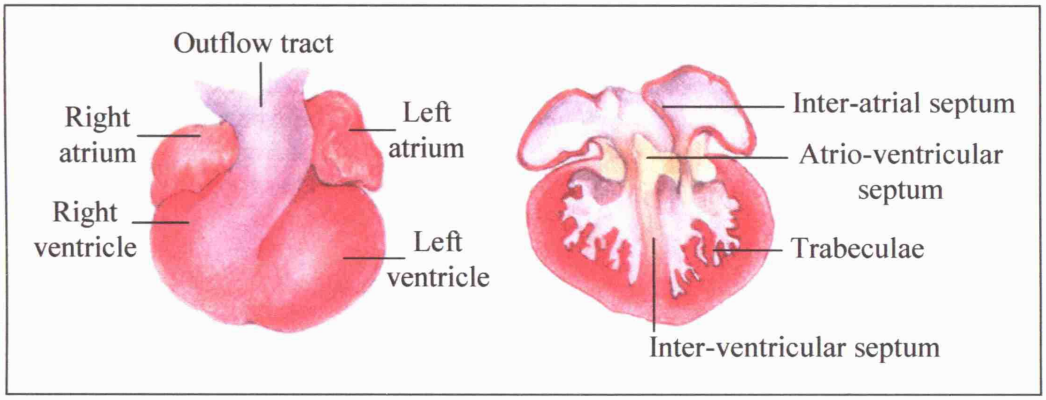


Figure 8. The remodelling heart. (Adapted from Harvey (2002). (Left) Isolated mouse heart at E12.5, and (right) representative frontal section. Myocardium is shown in red. Septation of the heart is completed so that all four chambers are distinct, and valves develop to ensure unidirectional blood flow. Trabeculae continue to form providing the heart with a contractile force. Further remodeling ensures the outflow tract becomes wedged ventrally between the two ventricles and the inflow tract is displaced dorsally. The vessels are now aligned correctly as in the adult heart.

Until recently, the cardiac chambers were said to arise from specific segments arranged along the anteroposterior axis of the linear and looping heart tube (Figure 1.9).

Christoffels et al (2000) have proposed the 'ballooning model' (Figure 1.9), a two-step model for chamber formation based on extensive gene expression studies. The first step is the formation of the linear heart tube, composed of 'primary' myocardium, which has polarity in phenotype and gene expression along the anteroposterior and dorsoventral axes. The second step is specification of 'secondary' (working or chamber) myocardium that has a different transcriptional program, at the ventral surface of the heart tube. As the heart tube loops, the primitive chambers are aligned facing the outer curvature of the heart, from where the chambers proliferate or 'balloon' out from the heart tube (Figure 1.9)(Christoffels et al., 2000). Genes are activated in the outer curvature demarcating the future working myocardium of the atria and ventricles including *atrial natriuretic factor* (*ANF*), *Chisel* and *Connexin 40* (*Cx40*) (Christoffels et al., 2000). The myocardium of the inner curvature, inflow tract, atrioventricular canal and outflow tract, retains the molecular signature it had in the linear heart tube, do not

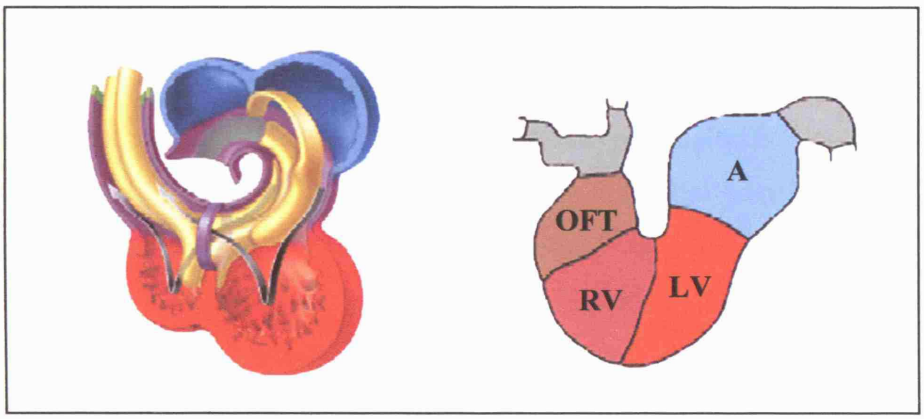


Figure 1.9. The 'ballooning' model of chamber morphogenesis versus segmentation. (Adapted from Christoffels et al., 2000). (Left) As the heart tube loops, the atrial (blue) and ventricular (red) chambers become positioned at and 'balloon' out from the outer curvature of the heart tube, differentiating into secondary, or working myocardium. The inner curvature of the heart, the outflow tract, inflow tract and atrioventricular canal retain the characteristics of the primary myocardium of the linear heart tube. (Right) Until recently, the heart tube that was said to be patterned along the anterior-posterior axis into segments that would ultimately form the various regions and chambers of the looped and mature heart. A, atria; LV, left ventricle; OFT, outflow tract; RV, right ventricle.

express the markers of working myocardium, and remain primary myocardium. Tbx2 prevents chamber formation in these regions of primary myocardium by repressing chamber myocardium specific genes such as *ANF* and *Cx40* (Habets et al., 2002; Christoffels et al., 2004).

Following chamber expansion, there is a requirement for separation of the chambers to ensure unidirectional blood flow. This is first established by the formation of the endocardial cushions, which are the precursors of the valves and membranous septae (Eisenberg and Markwald, 1995), at two points along the heart tube, the atrioventricular canal (AVC) and the outflow tract (OFT) (Figure 1.7). They first become apparent as acellular swellings in the cardiac jelly, which are subsequently invaded by endothelial cells at around E9.5. The endothelial cells that migrate into the cushions under-go an epithelial to mesenchymal transformation (EMT). As the cushions enlarge, they fuse and form the presumptive septal and valvular structures that will separate the developing atria, ventricles and outflow tract. The mesenchymal cells then become myocardial. Myocardialisation occurs in the mouse AVC and OFT from E12 and E13 onward respectively (van den Hoff et al., 1999). Myocardialisation is thought to occur by migration of existing cardiomyocytes from an epithelial position into the cushion mesenchyme, where they induce the mesenchymal cells to differentiate into cardiomyocytes (van den Hoff et al., 2004). However, the relative proportions of migration and differentiation are unknown and seem to differ between regions, and fate map analyses are being undertaken to better understand the myocardialisation of the cushions *in vivo* (van den Hoff et al., 2004). Transcription factors known to play a role in valve formation include Smad6 (Srivastava and Olson, 2000). Smad proteins transduce signals initiated by Tgfβ superfamily ligands. Hyperplasia of the cardiac valves and outflow tract septation defects in *Madh6* (encoding Smad6) mutant mice indicate a function for Smad6 in the regulation of endocardial cushion transformation (Galvin et al., 2000). Notch pathway mutants also have defects in valve formation due to impaired endocardial EMT (Timmerman et al., 2004).

The common atria and common ventricle are divided by formation of interatrial and interventricular septa, respectively. The growing endocardial cushions also contribute to the correct alignment of these developing septa.

In parallel with endocardial cushion formation, the ventricular myocardium grows out from the ventral surface of heart tube, and forms trabeculae that are finger-like

projections of myocardium that provide the contractile force to the developing heart. Trabeculae formation depends upon the neuregulin/Erbb signalling pathway (Harvey, 2002). From E12.5 the heart is remodelled. The chambers complete septation and the vessels are orientated to bring the outflow region in between the developing ventricles at the ventral surface, and the inflow region to the dorsal side of the heart (Harvey, 2002).

1.1.3.6 The outflow tract (OFT)

The outflow tract myocardium is derived from the anterior heart field (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). The outflow tract also receives a significant contribution from the cardiac neural crest (Cserjesi et al., 1995). The cardiac neural crest is a migratory population that arises from rhombomeres 6-8 (mid-otic placode to somite 3), and migrate through pharyngeal arches 3, 4 and 6, and from there a sub-population migrates into the outflow tract of the heart at E9.0, populating the conotruncus, aortic arches and proximal pulmonary arteries (Srivastava et al., 1997). The cardiac neural crest is involved in separation of the outflow tract into the aorta and pulmonary artery, and subsequent alignment of these great arteries. Neural crest cells are also involved in formation of the interventricular septum, separating the pulmonary and systemic circuits, and development of the semilunar valves. Neural crest cells additionally contribute to the aortic arch arteries, a region that accounts for up to 30% of CHDs (Srivastava and Olson, 2000).

Ablating the cardiac neural crest in chicks produces many cardiac defects, particularly defects of the outflow tract such as persistent truncus arteriosus, double-outlet right ventricle and aortic arch anomalies (Kirby and Waldo, 1995). Additionally, myocardial disfunction develops around stage 14 in the chick, before the neural crest cells migrate into the heart (Waldo et al., 1996), as an indirect effect of inadequate supply of growth factors in the pharyngeal region, which is itself normally regulated by the neural crest. This suggests that neural crest cells may play a role in myocardial maturation, independent of their role in septation of the outflow tract (Li et al., 2003).

The cardiac neural crest has been shown to be essential for the addition of myocardium, but not smooth muscle, to the outflow tract from the anterior heart field (Waldo et al., 2005a). In cardiac neural crest ablated chick embryos, the anterior heart field progenitor population proliferates at a greatly elevated level but fails to migrate into the

outflow tract and differentiate into myocardium, remaining instead at the junction of the OFT with the ventral pharynx (Waldo et al., 2005a). Consequently the outflow tract does not elongate, nor move caudally, resulting in abnormal connections with the aortic arch arteries.

1.1.3.7 Conduction system

Once septation of the chambers and valve formation have begun, specialised myocytes congregate to form the nodes (where the electrical impulse for contraction initiates) and the conduction system. However, the sinoatrial (SA) node, the pacemaker of the heart, can be first observed as a region of spontaneous depolarisation in the caudal part of the linear heart tube, even before contraction is visible (Olson and Srivastava, 1996). Initially, the electrical impulses spread from the SA node through the atria, and into the ventricles via the atrioventricular canal. As the endocardial cushions grow and define the atria and ventricles, a region of the interventricular septum forms the atrioventricular (AV) node providing the only depolarisation pathway between the atria and ventricles. The bundle of his and the Purkinjie fibres form to provide depolarisation to the ventricles. Transcription factors Nkx2.5 and Tbx5 have been implicated in conduction system formation as regulators of the Cx40 gap junction protein (Bruneau, 2002). Accordingly, *Nkx2.5*-deficient neonatal mice develop AV conduction block, and patients with *Nkx2.5* mutations suffer from progressive AV block (Pashmforoush et al., 2004). Tbx3 is thought to be involved in repressing *ANF* and *Cx40*, thereby repressing the development of chamber myocardium within the regions of the heart that will form components of the central conduction system of the heart (Hoogaars et al., 2004). This is coherent with the nodes and bundle branches of the conduction system having less well-developed contractile apparatus reminiscent of primary myocardium (Moorman and Christoffels, 2003).

1.1.4 Human heart development

Heart development in humans is similar to that described above in mouse. The cardiogenic plate is initially specified at day 15 and is equivalent to the cardiac crescent at E7.5 in mice. Two endocardial tubes then form on either side of the embryo, each with separate posterior inflow and anterior outflow regions. The endocardial tubes then fuse at the midline in a cranial to caudal fashion, as the embryo folds, resulting in a

single linear heart tube by day 21 that begins to contract spontaneously, which is equivalent to E8 in murine heart development. The heart tube then loops at around day 28 (E8.5 – E9.5 in mice) and begins to expand. The atria separate before the ventricles resulting temporarily in a three-chambered heart, similar that observed in the frog. By day 50, the four chambers and the outflow tract have completely septated, and the valves and conduction system are formed. Although there are subtle differences between human and murine heart development, there is a degree of evolutionary conservation such that many processes of cardiac morphogenesis, and the transcription factors that regulate them, are comparable between the species. It is hoped, therefore, that studying the development of the murine heart and the transcription factors involved may be able to provide some insight into the anomalies and factors underlying congenital heart disease in humans.

1.1.5 Summary

Heart development involves a complex series of precisely orchestrated molecular and morphogenetic events from the specification of precursors to the mature four-chambered organ. Many cellular processes are required, including cell movement, migration, specification and differentiation, governed for the most part by a large number of transcription factors, more of which are continually being identified. However, it is still little understood how such factors control their downstream targets and how the resulting molecular pathways translate into the morphogenetic events that occur during normal cardiogenesis. One such transcription factor is Hand1, a bHLH transcription factor that has been implicated in various stages of cardiac morphogenesis.

1.2 The bHLH transcription factor, Hand1

1.2.1 The bHLH transcription factor paradigm

Hand1, previously known as *Hxt*, *eHand* and *Thing1* (Cross et al., 1995; Cserjesi et al., 1995; Hollenberg et al., 1995) encodes a basic helix-loop-helix (bHLH) transcription factor. Transcription factors containing the bHLH domain have been shown to play a role in cell-lineage determination, and in regulating cell- and tissue-specific gene expression. For example *MyoD, myogenin, mrf-4* and *myf-5* are involved in mammalian skeletal muscle development, (Olson and Klein, 1994; Weintraub, 1993).

The bHLH domain itself consists of an HLH region of two amphipathic α-helices joined by a variable loop region, and a basic region, (a cluster of around 15 basic amino acids). Typically, bHLH transcription factors function as heterodimers, which dimerise via their HLH region. This dimerisation of proteins brings the two basic regions together resulting in the formation of a bipartite DNA binding domain that recognises and binds, typically, to the consensus E-box palindrome CANNTG. The classic paradigm is that lineage specific class B bHLH factors (including MyoD and Hand1) heterodimerise with the more ubiquitous class A bHLH factors such as the E-factors E12 and E47. In accordance with this paradigm, Hand1 has been shown to bind preferentially to a degenerate E-box, Thing1 (Th1) box, consensus sequence NNTCTG, as a heterodimer with widely expressed E12/E47 *in vitro* (Hollenberg et al., 1995). However, in contrast to the classic bHLH paradigm, Hand1 is also able to form heterodimers with other lineage restricted Class B bHLH factors such as Hand2 and hairy-related transcription factors (Hey/Hrt) (Firulli et al., 2000), and also with non-bHLH factors such as FHL2 (Hill and Riley, 2004) and Mef2 (Morin et al., 2005). Furthermore, Hand1 is able to form homodimers albeit with a lower affinity than Hand1 heterodimers (Firulli et al., 2000; Scott et al., 2000), and these homodimers have been shown to be functional at least *in vitro* (Hollenberg et al., 1995; Scott et al., 2000; Hill and Riley, 2004). Hand1 can function as a transcriptional activator or repressor, dependent on cell type, protein partner and target sequence. Hand1 has been shown to function as a transcriptional activator as a heterodimer with E12/E47 (Hollenberg et al., 1995), and also as a repressor by both direct inhibition of transcriptional activity and also by competing for protein binding partner (Bounpheng et al., 2000; Firulli et al., 2000; Scott et al., 2000).

Hand1 may also function independently of DNA binding since a *Hand1* transgene can phenocopy the preaxial polydactyly mediated by the HLH domain (minus the basic region) of Hand2 (McFadden et al., 2002).

1.2.2 Identification of Hand1

The murine Hand1 protein was first isolated by both interaction cloning (Cross et al., 1995) and yeast two-hybrid screens (Cserjesi et al., 1995; Hollenberg et al., 1995) using Class A bHLH proteins as bait. The screens also identified the closely related Hand2 protein (Cross et al., 1995; Hollenberg et al., 1995). The two proteins are most alike in the bHLH region with 87% amino acid homology (Cross et al., 1995) and are often described as being in the Twist sub-family of bHLH transcription factors (Figure 1.10). The two genes reside on separate chromosomes (Cross et al., 1995), and have different, but overlapping, expression patterns (Cross et al., 1995)(see below)(Cserjesi et al., 1995; Hollenberg et al., 1995; Srivastava et al., 1997). They are partially co-expressed in the initial stages of development, and later become restricted to specific regions.

It has been suggested that because *Hand1* and *Hand2* share high homology between their bHLH regions, and also have similar exon and intron organisation, the two arose from a duplication event of an ancestral *Hand*-like gene (Knofler et al., 1998). Phylogenetic analyses have placed the fish *Hand* into the same branch as the mammalian *Hand2*, which implies that *Hand2* is the ancestral form, but whether *Hand1* arose from a gene duplication event from *Hand2* remains to be elucidated (Kolsch and Paululat, 2002).

The human *Hand1* and *Hand2* genes reside on chromosomes 5q33 and 4q33 respectively. Chromosome 4q and 5q share over 13 pairs of paralogous genes, and it has been suggested that part of 5q arose from 4q in a tetraploidisation event resulting in the high level of similarity between these two chromosomal regions (Lundin, 1993). This provides further support for the model by which *Hand1* arose from *Hand2* by a duplication event (Natarajan et al., 2001).

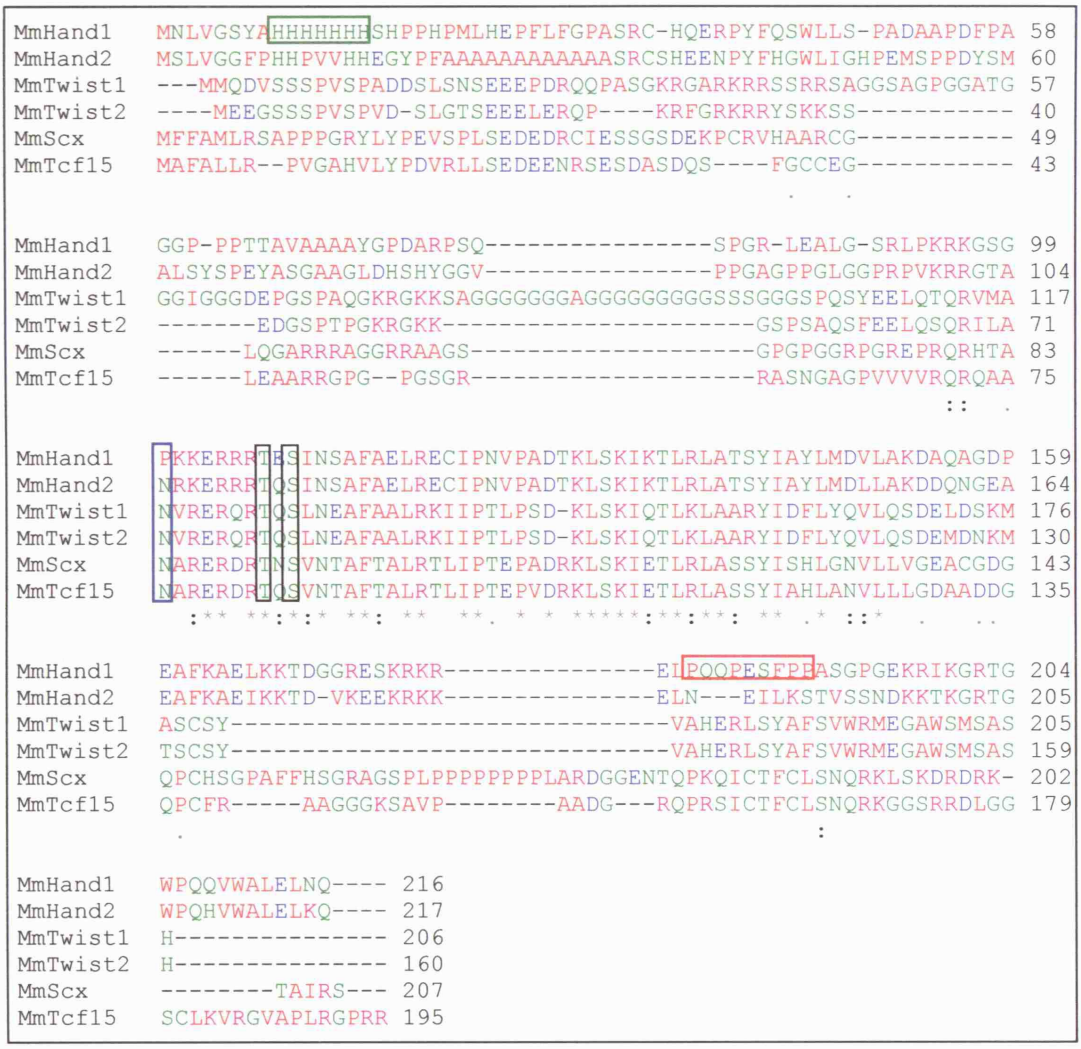


Figure 1.10. Hand proteins belong to the Twist sub-family of bHLH transcription factors. The mouse Hand1 protein sequence was aligned with Hand2 and selected members of the Twist sub-family of bHLH transcription factors, Twist1, Twist2, Scleraxis (Scx) and Transcription factor 15 (Tcf15). Conservation is observed within the bHLH region. The alignment also highlights the histidine rich region located in the N-terminus of Hand1 (green box), and the proline rich region in the C-terminus (red box), and further demonstrates the asparagine to proline substitution present in the basic region of the Hand1 bHLH domain (blue box). Also shown are the conserved serine and threonine residues in helix 1 of the bHLH domain (black boxes), phosphorylation state of which can affect subsequent dimerisation affinity. Amino acids that have similar properties have been grouped and colour coded. Conservation is as indicated; * absolutely conserved between sequences; : conserved substitutions based amino acid properties; . semi-conserved substitutions. The alignments were produced using the Clustal W program version 1.82 (http://www.ebi.ac.uk/clustalw/). Mm, *Mus musculus*.

1.2.3 Structure of Hand1

The murine *Hand1* gene is located on (proximal) chromosome 11 (Cross et al., 1995) and consists of two exons separated by a single 1.5 kb intron (Figure 1.11). The *Hand1* transcript is 1.9 kb and encodes a protein of 216 amino acids. The Hand1 protein has a histidine rich region in the N-terminus and a proline-rich region at the C-terminus (Figure 1.10)(Cserjesi et al., 1995).

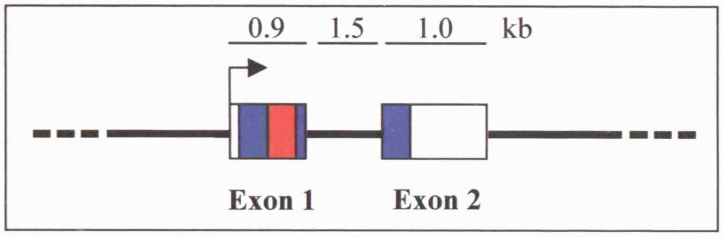


Figure 1.11. The structure of the *Hand1* gene. The *Hand1* gene consists of two exons of 0.9 kb and 1.0 kb in length, separated by a single 1.5 kb intron. The coding regions are shown in blue and non-coding regions are shown in white. The region encoding the bHLH domain is shown in red.

The bHLH domain of Hand1, and also that of Hand2 and Hand proteins, is highly conserved across species, as is a C-terminal domain of around 16 amino acids that has been termed the Hand-domain and has an undetermined role (Figure 1.12)(Kolsch and Paululat, 2002).

Hand1 has low sequence identity to most other bHLH proteins, being most closely related to HEN1 (Brown and Baer, 1994), which is expressed in the central nervous system and regulates neurogenesis and neuronal differentiation (Bao et al., 2000). One of the most striking structural differences is in the basic region where a proline residue replaces an asparagine that is highly conserved throughout bHLH factors and is important for DNA binding (Figure 1.10). The asparagine to proline substitution in the basic domain is normally associated with transcriptional repression, being present in the Drosophila bHLH factors enhancer-of-split (E(Spl)) and hairy (h) (Garrell and Campuzano, 1991). The presence of the histidine rich domain in the N-terminus of Hand1 (Figure 1.10) is also suggestive of repressive function, although the role of this domain in Hand1 has not been investigated. In support of a role for Hand1 in negative regulation, there is *in vitro* evidence of Hand1 acting as a repressor through several

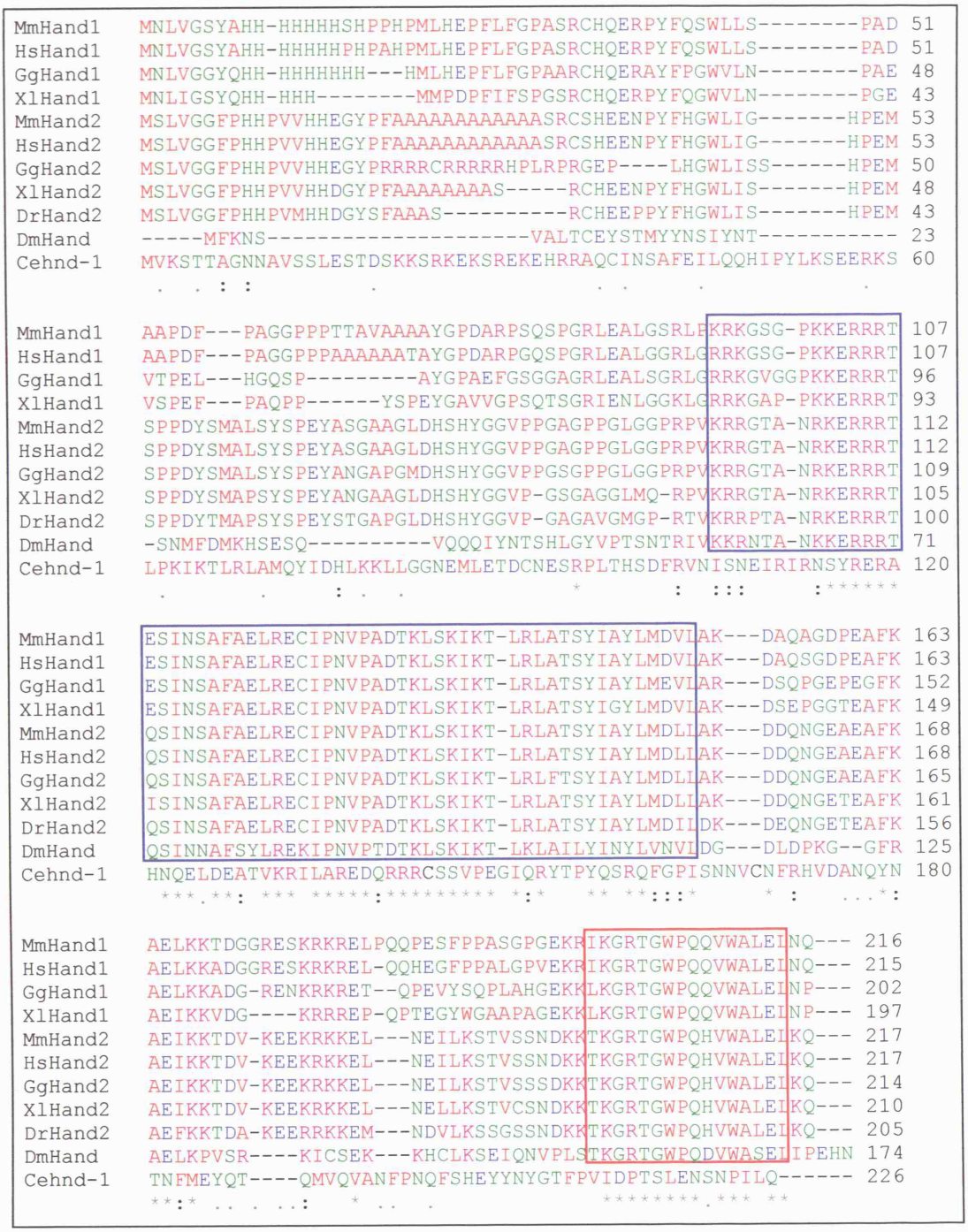


Figure 1.12. The Hand proteins are conserved between diverse species. Hand1, Hand2 or Hand protein sequences from mouse, human, chick, frog, zebrafish, fly and nematode worm were aligned as in Figure 1.10. The conservation between the species is most striking in the bHLH domain (blue box) and the so-called Hand domain (red box). Indication of conservation excludes *C. elegans* sequences. Mm, *Mus musculus*; Hs, *Homo sapiens*; Gg, *Gallus gallus*; Xl, *Xenopus laevis*; Dr, *Danio rerio*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*.

mechanisms. Hand1 has been shown to sequester class A bHLH factors from E-boxes (Knofler et al., 2002), to antagonise Mash2 function by competing for E-factor binding (Scott et al., 2000), and also to directly inhibit transcriptional activity of tethered MyoD/E12 heterodimers (Bounpheng et al., 2000).

1.2.4 Regulation of Hand1 activity

Hand1, despite being a class B tissue-specific bHLH transcription factor, is expressed in a variety of cell types. Accordingly, Hand1 appears to impact on many genetic pathways and can have both a positive and negative effect on downstream target genes. Until recently, relatively little was known about the regulation of Hand1 activity, but given the factors discussed above, it was assumed to be relatively complex. Moreover, in addition to the broad range of Hand1 dimerisation partners in the bHLH family of proteins, Hand1 has also been shown to interact with non-bHLH proteins, the LIM-domain containing protein, FHL2 (Hill and Riley, 2004), the MADS-domain containing Mef2 proteins (Morin et al., 2005) and possibly GATA factors (Morin et al., 2005). Recently, further levels of complexity have been added to Hand1 dimerisation and the regulation of transcriptional activity that show that Hand1 dimerisation choice can be controlled by phosphorylation (Firulli et al., 2003), and that tertiary co-factors can affect activity after the initial dimerisation choice has been made (Hill and Riley, 2004).

1.2.4.1 Regulation of Hand1 activity by phosphorylation

B56δ is a regulatory subunit of the protein phosphatase 2A (PP2A) and was identified as a Hand1 interacting protein in a yeast two-hybrid assay (Firulli et al., 2003), suggesting that Hand1 dimerisation and/or biological function may be regulated by post-translational modification by phosphorylation. It has been shown that the phosphorylation state of two conserved residues (one serine, one threonine) in helix 1 of the bHLH domain (Figure 1.10) is controlled by protein kinase-A and -C (PKA, and PKC) and PP2A, and that phosphorylation state influences the dimerisation affinity of Hand1 with other bHLH factors, which in turn can affect biological activity within the cell (Firulli et al., 2003).

The two residues in helix 1 of the bHLH domain are not only highly conserved between Hand proteins, but are also highly conserved amongst the bHLH domains of Twist family proteins (Figure 1.10), including Twist1 homologs as distant as *Drosophila*

Twist (Firulli et al., 2005). This suggests that control of bHLH activity by post-translational modification by phosphorylation is evolutionarily conserved between the Twist family of bHLH factors. Firulli and co-workers (2005) have shown that phosphorylation of these conserved residues in two other family members, Twist1 and Hand2, affects dimerisation affinity, and that mutations in human Twist1 that affect the phosphorylation state of the conserved residues are responsible for causing Saethre-Chotzen syndrome, presumably by altering Twist1 dimerisation affinity. Dimerisation affinity was also shown to be dose-dependent (Firulli et al., 2005), as seen in many instances, where relative abundance of proteins in the dimerisation pool can affect the choice of protein partner.

1.2.4.2 Tertiary regulation of Hand1 activity

FHL2 is a member of the four-and-a-half LIM domain protein family and has been shown to repress Hand1/E12 heterodimer co-activation of downstream target genes, but has no effect on Hand1 homodimers (Hill and Riley, 2004). Furthermore, it was shown that the inhibition of Hand1/E12 was not a consequence of dimerisation interference as Hand1/E12-tethered heterodimers were also repressed by FHL2, and neither does FHL2 interfere with heterodimer DNA binding as shown by EMSAs. The interaction with FHL2, therefore, seems to regulate Hand1 activity subsequently to dimerisation choice.

1.2.5 Expression of *Hand1*

1.2.5.1 Expression of *Hand1* in the heart and extra-embryonic tissues

The expression of the *Hand1* gene is both stage and tissue-specific. Unlike most other class B bHLH transcription factors, it is expressed in a wide range of tissues in a complex pattern, and not restricted to a single lineage. Much of the cardiovascular system is derived from lateral mesoderm and cardiac neural crest, and *Hand1* is expressed at relatively high levels in both of these key cell types.

Hand1 expression can be first detected in extra-embryonic tissues at E7.5 (Cross et al., 1995; Cserjesi et al., 1995; Hollenberg et al., 1995) and expression remains high in these tissues throughout embryonic development. *Hand1* is initially expressed in the trophoectoderm and subsequently throughout the trophoblast of the developing placenta. Expression is high in the ectoplacental cone but is restricted to the more

differentiated trophoblast cells at the maternal-foetal interface known as trophoblast giant cells. *Hand1* is also transiently expressed in other extra-embryonic lineages such as the allantois and the amnion.

At later stages in the embryo proper, *Hand1* is expressed in the embryonic cardiac and neural crest cells, along with some other neural crest derived tissues (Cross et al., 1995; Cserjesi et al., 1995; Hollenberg et al., 1995). In the heart, *Hand1* expression is first detected throughout the cardiac progenitors of the cardiac crescent. At this point, Hand1 is also found in the surrounding pericardium and the peripheral regions of the distal lateral mesoderm (Cserjesi et al., 1995). *Hand1* is initially expressed at the ventral surface, in the anterior and posterior portions of the linear heart tube, symmetrically along the LR axis, in the aortic sac, conotruncus, left ventricle and atrial-forming regions of the linear heart tube. *Hand1* is not expressed in the intervening region. As the heart tube loops, expression becomes left-sided. By E9.5 after cardiac looping, *Hand1* expression is limited to the outer curvature of the left (systemic) ventricle and the neural crest derived developing outflow tract (Figure 1.13)(Srivastava et al., 1995). Expression remains high in the developing heart until E10.5, with weak expression observed additionally in the presumptive right ventricle, after which it declines. At E11.5 *Hand1* is still expressed in the outflow tract, and by E13.5 expression becomes restricted to the regions of future valve development, gradually becoming more confined to the distal portions, or the growing edge of these regions (Cserjesi et al., 1995).

Figure 1.13. *Hand1* expression in the mouse embryo at E9.5. At E9.5, subsequent to cardiac looping, *Hand1* expression in the developing mouse heart becomes restricted to the outer curvature of the left ventricle (lv) and the outflow tract (oft). At this stage, *Hand1* is also expressed in the lateral plate mesoderm (lpm) and at the midline region of the first branchial arch (ba).

1.2.5.2 Expression of *Hand1* in other lineages

After E9.5, *Hand1* expression is limited to regions of the embryo that have a large neural crest contribution (Hollenberg *et al.* 1995). At E10.5 there is expression in the precursors of the sympathetic trunk ganglia, and it continues to be expressed in this lineage until at least E14.5, peaking at E13.5. By E11.5, high levels of expression are also seen in the first pharyngeal arch, and the developing mid- and hindgut (also lateral mesoderm derivatives), all of which are structures that have a high neural crest derived component (Cserjesi et al., 1995). *Hand1* is expressed in the head at E12.5, in the septal region of the tongue bud and in the mandible. By E14.5 expression levels are beginning to drop off, with some expression maintained in the sympathoadrenal lineage, intestinal system and adrenal medulla (Cserjesi et al., 1995).

Initially, only low levels of transient expression were observed in distal posterior regions of the limb buds at E10.5 (Cserjesi et al., 1995). Fernandez-Teran et al (2003) have since reported a dynamic expression pattern for *Hand1* in the developing limb but with consistent expression in the anteroventral regions (Fernandez-Teran et al., 2003).

1.2.5.3 Expression of *Hand1* in adult mice

Hand1 expression in the adult mouse has been detected in the smooth muscle cells of the gut and in the liver by RT-PCR (Hollenberg et al., 1995). Expression has also been detected in the adult brain, muscle and testis (Cross et al., 1995), and intestine and adrenal tissue (Cserjesi et al., 1995). Initially, it was reported to be absent from the adult mouse heart (Cserjesi et al., 1995; Srivastava et al., 1995). However, recently it has been shown that Hand1 is expressed in the left and right ventricles of adult mouse (and rat) hearts, but is absent from the atria (Thattaliyath et al., 2002a).

1.2.5.4 Human *Hand1* expression

Human *Hand1* is highly expressed in adult heart (Knofler et al., 1998), *Hand1* is expressed strongly in both the right and left ventricles, but is barely detectable in both atria (Natarajan et al., 2001). The human *Hand1* expression pattern was thought to differ from murine *Hand1* expression as murine *Hand1* was initially only detected in the developing embryonic heart (Cserjesi et al., 1995). However, *Hand1* expression has now been illustrated in adult rodent heart (Thattaliyath et al., 2002a), which is more in

keeping with the high degree of molecular and functional conservation of Hand1 across the species.

Hand1 is also expressed in human extra-embryonic tissues, in trophoblast-like cells and the amniotic epithelium suggesting that it may have analogous function at some stage of extra-embryonic development as murine Hand1 (Knofler et al., 1998).

1.2.5.5 *Hand* gene expression in other species

Hand genes, and their expression in mesodermal populations and in the developing heart, are evolutionarily conserved between species (Figure 1.12). Homologs have been identified in ascidians, *C. elegans*, *Drosophila*, Zebrafish, *Xenopus*, chick, mouse and humans (Dehal et al., 2002; Mathies et al., 2003; Kolsch and Paululat, 2002; Angelo et al., 2000; Sparrow et al., 1998; Srivastava et al., 1995; Cross et al., 1995; Knofler et al., 1998; Russell et al., 1997; Russell et al., 1998). Humans and higher vertebrates have two *Hand* genes, whereas only a single hand gene has been identified in ascidians, *C. elegans*, *Drosophila* and zebrafish.

The chick *Hand1* and *Hand2* have very similar expression patterns, and also bear similarity to their expression in mouse (Srivastava et al., 1997). *Hand1* is first detected at Hamburger and Hamilton (HH) stage 8 in the cardiac crescent, and in the paired heart primordia as they fuse at HH stage 9. *Hand1* is expressed throughout the cardiac tube and the inflow region at HH stage 10. At HH stage15, after cardiac looping, *Hand1* is expressed in the atria, the future left ventricle, bulbus cordis, truncus arteriosus and throughout the branchial arches. It has been suggested that the chick Hand1 and Hand2 are functionally redundant (see sections 1.2.6.1 and 1.2.8).

The single *hand* gene in *Drosophila* is expressed throughout the dorsal vessel, in both the cardioblasts and the pericardial cells, and is not more closely related to either *Hand1* or *Hand2* (Kolsch and Paululat, 2002). In addition it is expressed in the progenitors of the circular visceral muscles, the lymph gland, garland cells and a few cells in the CNS. *Drosophila hand* is first detected after the tissues have been specified, indicating a role in differentiation rather than tissue specification. A minimal cardiac and haematopoietic enhancer has recently been identified for *Drosophila hand.* It has also been shown that Tinman (homolog of NK-type homeobox transcription factors) and the GATA factors Pannier and Serpent that control cardiogenesis and haematopoiesis directly activate this

enhancer via conserved tinman and GATA binding sites (Han and Olson, 2005). This prompted the suggestion that GATA factors also regulate *Hand* gene expression in vertebrates (Han and Olson, 2005) since two essential GATA-binding sites have been identified in the murine *Hand2* enhancer region (McFadden et al., 2000). Given the high level of transcriptional conservation in heart development, the fact that the enhancer identified is responsible for both cardiovascular and haematopoietic *Drosophila hand* expression implicates vertebrate *Hand* genes in haematopoietic development in addition to cardiogenesis, and further suggests that the processes of cardiogenesis and haematopoiesis are more closely linked than previously thought (Han and Olson, 2005).

There is a single *Hand* gene in the Zebrafish genome, *han*, that is also expressed throughout the heart. Unlike *Drosophila Hand*, *han* appears to be more closely related to *Hand2* (Angelo et al., 2000). The *han* mutant, *hands off*, has severe cardiac defects at an early stage of heart development, and have a reduced number of cardiac precursors (Yelon et al., 2000).

The *C. elegans Hand* gene, *hnd-1*, is first broadly expressed in the mesoderm and then becomes restricted to the somatic gonadal precursor cells (SGP)(Mathies et al., 2003). *hnd-1* appears to be important for embryonic viability potentially through controlling muscle cell fate, and also to be responsible for maintaining SGP fate and survival. Again, *hnd-1* is implicated not in specification but in subsequent differentiation. The role of *hnd-1* in maintaining precursor cells has also been likened to the role of *han* in zebrafish in maintaining the correct number of cardiac precursor cells (Mathies et al., 2003).

It appears that the Hand proteins play a conserved role in cardiogenesis, with an emphasis on regulating differentiation of cardiomyocytes rather than the initial specification event.

1.2.6 Function of Hand1 during murine development

1.2.6.1 Introduction

The first insight into Hand1 function arose from over-expression studies in mice (Cross et al., 1995) and antisense experiments in chicks (Srivastava et al., 1995).

Hand1 was over-expressed in mouse blastomeres by injecting an expression plasmid into the nucleus of an individual cell in mouse two-cell embryos, and the embryos cultured until the blastocyst stage when they were assessed. It was found that Hand1 promoted trophoblast differentiation and arrested cell growth (Cross et al., 1995). Further experiments in Rcho-1 (rat choriocarcinoma) cells showed that over-expression of Hand1 accelerated trophoblast giant cell differentiation and that *Hand1* expression is up-regulated coincident with differentiation following serum withdrawal (Cross et al., 1995; Scott et al., 2000). These experiments suggested that, at least in mice, Hand1 regulates cell commitment to the trophoblast lineage, and subsequent differentiation.

Antisense experiments in chicks (Srivastava et al., 1995) implied a role in heart development. Stage 8 chick embryos were treated with antisense oligonucleotides specific to *Hand1* and *Hand2* transcripts, both singly, and in combination with each other, to prevent their expression in the embryos. The single antisense oligonucleotides had no effect on cardiac development, whereas in combination the antisense oligonucleotides lead to arrest of cardiac morphogenesis at the point of cardiac looping (stage 11-12). At the time of arrest, the hearts had initiated rightward looping, and the primitive ventricular and atrial chambers had been specified. Since cardiac development was only arrested when both oligonucleotides were present, it was suggested that Hand1 and Hand2 have redundant roles.

1.2.6.2 Hand1 is essential for placentation

The oligonucleotide experiments and over-expression studies indicated a role for Hand1 in heart development and rodent trophoblast cell differentiation respectively. Riley et al (1998) and others (Firulli et al., 1998) generated a knock-out of *Hand1* by gene targeting to gain further insight into Hand1 function *in vivo*. Mice heterozygous for *Hand1* have no apparent phenotype. *Hand1*-null embryos arrested at E7.5 due to extra-embryonic defects indicating that Hand1 is essential for placentation. More specifically, the data implied that Hand1 was necessary for differentiation of ectoplacental cone cells into trophoblast giant cells (Riley et al., 1998) and also for appropriate development of the visceral yolk sac vasculature (Firulli et al., 1998). Whilst confirming a role for Hand1 in trophoblast differentiation, the early lethality of *Hand1*-null mutants prevented analysis of Hand1 function in the other lineages in which it is expressed including the developing heart.

1.2.6.3 Hand1 is essential for cardiac morphogenesis

In order to prove the early arrest of *Hand1*-null mutants was caused by abnormal trophoblast function, and to investigate a role for Hand1 in the embryo proper, tetraploid-rescue was performed (Riley et al., 1998). Aggregation chimeras were produced from *Hand1* mutant and tetraploid morulae whereby the resulting embryo was derived from *Hand1* mutant cells and the extra-embryonic tissues were extensively derived from the wildtype tetraploid cells. Therefore, if the early lethality was primarily due to extra-embryonic defects, the embryo would be rescued and develop further, allowing analysis of Hand1 function in the embryo proper.

Tetraploid-rescued *Hand1*-null embryos arrested around E10.0. Partial rescue confirmed the extra-embryonic trophoblast phenotype of the homozygous mutants, however, the tetraploid rescued embryos died of heart failure. Cardiac looping did not occur in the rescued mutants, there was apparent pericardial oedema, no chamber distinction and no myocardial trabeculation leading to a thin ventricular wall suggesting, therefore, that Hand1 plays an essential role in cardiac morphogenesis (Riley et al., 1998).

Expression of myocardial marker genes were assessed and found to be normal in *Hand1*-null mutants (Riley et al., 1998). Hand1, therefore, did not appear to be required for differentiation of cardiomyocytes. However, *MLC2v* was undetectable, suggesting that Hand1 might be involved in specification of ventricular myocardium.

Similarly Hand2 has been implicated in specifying the right ventricle. *Hand2*-null embryos arrest after cardiac looping has initiated at E10.5, and specification of the right (pulmonary) ventricle does not occur (Srivastava et al., 1997). This, together with the *Hand2/inv* double mutants in which the left-sided pulmonary ventricle was not specified (Thomas et al., 1998), suggested a role for Hand proteins in specifying ventricular chamber identity. That said, it would appear that Hand1 function may be more complex than left ventricle specification as the null mutants arrest at a more primitive stage, at or around the initiation of looping (Thomas et al., 1998) prior to chamber specification.

1.2.6.4 Cardiomyocyte differentiation occurs in the absence of Hand1

Hand1-null ES cells, formed from sequential targeting of both *Hand1* alleles, were seen to differentiate into beating cardiomyocytes *in vitro* (Riley et al., 2000). Cardiac-

specific transcripts such as *Nkx2.5*, *MLC2a* and *MLC2v* were expressed in the differentiated *Hand1*-null cells implying that Hand1 is not necessary for ventricular lineage commitment or cardiomyocyte proliferation to occur (Riley et al., 2000). Moreover, this suggested that the thin ventricular wall and down-regulation of *MLC2v* seen in the tetraploid-rescued *Hand1*-null embryos (Riley et al., 1998) might be secondary effects.

1.2.6.5 Cell autonomous role for Hand1 during cardiac looping

Chimeric embryos were made from *Hand1*-null ES cells aggregated with ROSA26 embryos (Riley et al., 2000). In low contribution chimeras (below 50%), non-random distribution of *Hand1*-null cells was observed. *Hand1* mutant cells were excluded from the caudal region of the heart tube fated to become the left ventricle at E8.0. This exclusion implies that Hand1 is not wholly responsible for left ventricular specification as it is only required in the outer curvature, although it may still play a role in growth and maintenance of the ventricle (Riley et al., 2000). Significantly, cells in the left caudal region of the linear heart tube may be involved in the initiation of looping (Biben and Harvey, 1997; Taber et al., 1995). Furthermore, at E9.5 during overt looping, *Hand1*-null cells were excluded from the anterior ventral portion and outer curvature of the left ventricle (Riley et al., 2000). The outer curvature region has been identified as the leading edge of the heart tube during looping morphogenesis, derived from the left heart progenitor pool thought to respond to left-right signalling (Biben and Harvey, 1997). The chimeric studies collectively indicate a cell autonomous requirement for Hand1 in a subset of cardiac mesodermal derivatives that contribute to the outer curvature of the left ventricle and in a population of migratory cells actively involved in cardiac looping (Riley et al., 2000).

1.2.6.6 Hand1 function in neural crest differentiation

As discussed previously, neural crest cells are a migratory population of cells and specifically, cardiac neural crest is involved in septation of the outflow tract and also contribute to the interventricular septum (see section 1.1.3.6). *Hand1* expression in various neural crest derived tissues such as the outflow tract (Thomas et al., 1998) coincides with neural crest condensation at their terminal locations. This suggests Hand1 plays a role in neural crest differentiation. In support of this presumptive role and indicating a potential role in cell migration and/or commitment, *Hand1*-null cells

are excluded from pre-migratory crest in ROSA26/*Hand1*-null chimeras at E9.5 (Riley et al., 2000), as well as being expressed in pre-migratory populations of neural crest such as the sympathetic trunk progenitors (Cserjesi et al., 1995).

Both *Hand* genes are down-regulated in neural crest derived tissues in *Endothelin-1* (ET1) deficient mice (Olson and Srivastava, 1996). In the branchial arches, *Hand* gene expression is adjacent to that of *ET-1*, and adjacent to that of ET-1 receptor (ET_A) (Srivastava, 1999). The Hand transcription factors may, therefore, function in the same pathway as ET-1.

1.2.6.7 Hand1 and cell migration

The identification of a cell-autonomous role for Hand1 in migratory cardiomyocyte and neural crest populations (Riley et al., 2000) is suggestive of a role for Hand1 in cell migration. Appropriately, various genes known to be involved directly, or indirectly, in cell movement and migration were identified as putative targets of Hand1, including *Thymosin β4*, *Cystatin C* and *α-cardiac actin*, using representational differential analysis (RDA), which compared differentiated wildtype and *Hand1*-null ES cells, (Smart et al., 2002). The results of the RDA screen substantiate a putative role for Hand1 in cell migration in the developing heart. In addition to genes involved in cell migration, the RDA screen identified various other genes, including four involved in cell cycle regulation.

1.2.6.8 Hand1 function in interventricular septum formation

Two recent studies have shed light on a putative role for Hand1 in the formation of the interventricular septum. Firstly, *Hand1* was targeted to the *Mlc2v* locus by homologous recombination, which resulted in ectopic *Hand1* expression throughout the entire ventricular myocardium (left and right sided) including the intervening region that forms the interventricular septum (Togi et al., 2004). These *Hand1* knock-in mice die between E12.5 and E14.5, having a single, expanded ventricle, completely lacking an interventricular septum. In order to rule out that the septal defect was a secondary or non-specific effect, Togi and co-workers (2004) went on to generate transgenic embryos that over-expressed *Hand1* in the ventricles only, ensuring *Hand1* expression was absent from the boundary region between the two ventricles. In these transgenic embryos, the interventicular septum formed normally. This suggested that Hand1 is

normally involved in defining the ventricular boundary, and position of the interventricular septum, in a non-cell autonomous fashion. This study also implied that Hand1 is not directly involved in specifying 'leftness' to the left ventricle

In support of the presumptive role of Hand1 in septum formation, McFadden et al (2005) have generated a cardiac specific knock-out of *Hand1* utilising the Cre-*lox* system crossing both *Nkx2.5-Cre* and *α-MHC-Cre* mice with floxed *Hand1* mice. These knock-out mice die perinatally, most commonly displaying ventricular septal defects (VSD), atrioventricular (AV) valve defects and outflow tract defects. It was noted that the *Hand1* cardiac knock-out mice have a thickened, disorganised septum at all stages of development, implying that in the absence of Hand1, growth of or positioning of the interventricular septum is abnormal.

In combination these studies implicate Hand1 in interventricular septum formation. Over-expression of *Hand1* abolishes septum formation (Togi et al., 2004) whilst cardiac deletion of *Hand1* causes an expansion of the septum (McFadden et al., 2005).

1.2.6.9 Hand1 is involved in left ventricle and outflow tract development

In addition to implicating Hand1 in septum formation, ectopic expression of *Hand1* in the boundary region between the two presumptive ventricles, that would normally form the interventricular groove/septum, results in the formation of a single expanded ventricle, suggesting Hand1 is involved in ventricular expansion (Togi et al., 2004). In the subsequent cardiac knock-out study, the *Hand* genes were shown to act in a dosage-dependent manner in controlling left ventricle growth and expansion (McFadden et al., 2005). The left ventricle was reduced in size in *Hand1* cardiac knock-out embryos at E11.5 and E13.5, however this appeared to recover by birth. However, the combined *Hand1* cardiac knock-out/*Hand2* heterozygote resulted in embryonic lethality at E10.5 with a thin and poorly trabeculated myocardium. Accordingly, the expression of *ANF* was shown to be dependent on the levels of Hand1 and Hand2, being completely absent in the ventricles of *Hand1* cardiac knock-out/*Hand2* heterozygote embryos, reflecting the observed lack of ventricular chamber expansion. No change was detected in *Mlc2v* expression in either the *Hand1* cardiac knock-out embryos, or the *Hand1* cardiac knock-out/*Hand2* heterozygote embryos, suggesting that ventricular specification is not dependent upon Hand1 or Hand2.

Outflow tract defects were also observed in some of the *Hand1* cardiac knock-out mice at postnatal day 1-2 *(α-MHC*-Cre)(McFadden et al., 2005). Although McFadden and co-workers (2005) did not investigate the outflow tract defects further, they are indicative of a putative role for Hand1 in outflow tract morphogenesis. Both of these observations support previous studies indicating a role for Hand1 in growth and maintenance of the left ventricle and morphogenesis of the outflow tract (Riley et al., 2000); see section 1.2.6.5).

1.2.6.10 *ANF* is a downstream target gene for Hand1

The studies described above also suggested that *ANF* was downstream of Hand1 in the same pathway. *Hand1* mis-expression throughout the ventricular region resulted in an increase in *ANF* expression levels and ectopic expression in the inner curvature of the ventricles (Togi et al., 2004), whilst the cardiac specific knock-out of *Hand1* resulted in decreased *ANF* expression in the left ventricle, and completely absent in *Hand1/Hand2* double mutants (McFadden et al., 2005). Recently, *ANF* was shown to be a direct downstream target of Hand1 (Morin et al., 2005) in that Hand1 directly activated a cardiac specific *ANF* promoter in transfection studies, and furthermore, was shown to physically interact with Mef2 proteins to synergistically activate expression of *ANF* via *Mef2* binding sites.

1.2.6.11 Hand1 function in valve formation

Hand1 cardiac knock-out embryos also displayed abnormally thickened AV valves and hyperplastic endocardial cushions (McFadden et al., 2005). The authors propose that, because Cre expression is not driven in the endocardial cushions or cardiac valves, Hand1 is mediating a signal derived from the myocardium that regulates endocardial cushion morphogenesis, and that this signal is independent of the TGFβ- and BMP-mediated signals normally associated with signalling between the endocardium and myocardium. However, early myocardial function has been shown to affect endocardial cushion development (Bartman et al., 2004) and therefore abnormalities in cardiac contractility or morphogenesis in *Hand1* cardiac knock-out embryos could be having an indirect effect on endocardial cushion development.

1.2.7 Down-regulation of *Hand1* leads to cardiac hypertrophy

The expression of Hand1 in adult heart is suggestive of a putative role in the adult heart. Recently an additional function for Hand1 has been proposed, namely modifying cardiac responses in the myocardium to genetic insult or environmental stresses and mediating hypertrophic signalling. In support of this role, human *Hand1* has been seen to be dramatically down-regulated in the ventricles of patients with dilated or ischemic cardiomyopathy (Natarajan et al., 2001). Cardiac hypertrophy was induced pharmacologically in mice by treatment with phenylephrine, which resulted in chamber specific down-regulation of *Hand1* within the left ventricle, and *Hand2* within the right ventricle (Thattaliyath et al., 2002a). In the same study, hypertrophy was induced surgically, by aortic-constriction, in rats, resulting in down-regulation of both *Hand1* and *Hand2* in both ventricles. Although different stimuli produce different responses, the down-regulation of the *Hand* genes prior to cardiac hypertrophy is a common factor suggestive of a role in the inhibition of cardiac hypertrophy (Thattaliyath et al., 2002a).

The absence of the cardiac lineage protein-1 gene (*CLP-1*) results in a foetal form of cardiac hypertrophy (Huang et al., 2004). *CLP-1*-null embryos die from around E17.5 onwards presenting with a decreased left ventricular chamber size and a thickened left ventricular wall, accompanied by an up-regulation of beta-myosin heavy chain (*β-MHC*), *alpha-skeletal actin* and *ANF*. Re-expression of these genes is normally associated with an adaptive response to cardiac hypertrophy. Interestingly, *Hand1* was shown to be significantly down-regulated throughout cardiac development in *CLP-1*-null embryos, from E9.5 onwards prior to the onset of cardiac hypertrophy (Huang et al., 2004). Huang and co-workers (2004) have suggested that disruption of *Hand1* expression in early cardiac morphogenesis is the direct cause for the foetal hypertrophy in the later stages of development.

Furthermore, in mice deficient for Irx4, adult onset cardiomyopathy is preceded by significantly reduced expression of *Hand1* in the anterior and ventral regions of the developing left ventricle at mid-gestation stages (Bruneau et al., 2001). Collectively, these studies imply that Hand1 is involved in inhibiting cardiac hypertrophy in the adult heart, and that this role begins during embryonic development.

1.2.8 Functional redundancy of mammalian *Hand* genes

Initially, antisense studies in chick (see section 1.2.6.1) suggested that Hand1 and Hand2 had redundant roles in cardiac development (Srivastava et al., 1995). However, the distinct, partially overlapping expression patterns in mice and the phenotypes of *Hand1*-null and *Hand2*-null embryos suggest that Hand1 and Hand2 have separate and independent roles in identifying left or right ventricular chamber identity respectively (Thomas et al., 1998); see section 1.2.6.3).

The zebrafish has a single *Hand* gene, *hands off* (*han*), which is homologous to murine *Hand2*. Deletion of the *hands off* gene results in severe cardiac defects at an early stage of heart development (Yelon et al., 2000). *hands off* mutants have a reduced number of cardiac precursors, and the myocardial tissue that does form is improperly patterned, developing as two small, lateral clusters that do not fuse at the midline, and that are primarily atrial in phenotype. Because of the difference in severity of the mutant phenotypes in mice and zebrafish, it was suggested that in mouse, Hand1 might be compensating for loss of Hand2 in *Hand2*-null mutants, since their expression patterns do partially overlap (Yelon et al., 2000). Since only one *Hand* gene has been identified in zebrafish, no such compensation can occur, and the resultant phenotype is accordingly, much more severe.

Furthermore, loss of a single copy of *Hand2* (*Hand2* heterozygote) exacerbated the *Hand1* cardiac knock-out phenotype resulting in embryonic lethality at around E10.5 (McFadden et al., 2005). In isolation, *Hand1* cardiac knock-out mice die in the perinatal period and *Hand2* heterozygotes are viable. Equally, embryos with one copy of *Hand1* on a *Hand2*-null background were delayed and died around E9.5. Loss of both copies of *Hand2* (*Hand2*-null) in combination with the *Hand1* cardiac knock-out further intensified the severity of the phenotype causing embryonic lethality at around E9.0 (McFadden et al., 2005). This is highly suggestive of *Hand* gene compensation in the developing heart, although must be considered in the context of Cre expression in the two Cre mouse strains used in this study. Additionally, in mice, over-expression of the HLH domain of either Hand1 or Hand2 in the developing limb bud induces polydactyly, demonstrating functional similarity in biological activity (McFadden et al., 2002). The functional over-lap of the *Hand* genes could be explained in part by their having a common dimerisation partner that retains a dominant function regardless of to which Hand protein it is bound (Thattaliyath et al., 2002a).

However, the *Hand* genes do have distinct, non-overlapping functions in the heart. Hand2 and Nkx2.5 have been shown to synergistically regulate *ANF*, but Hand1 and Nkx2.5 do not despite Hand1 and Nkx2.5 interacting physically (Thattaliyath et al., 2002b).

Similarly, in the extra-embryonic membranes, Hand1 and Hand2 appear to regulate vascular development through separate pathways, as up-regulation of *Hand2* does not rescue the *Hand1*-null yolk sac defect (Morikawa and Cserjesi, 2004). It seems, therefore, that the Hand proteins are able compensate the loss of the other to a degree, but that not all aspects of Hand1 and Hand2 function are redundant.

1.2.9 Hand1 function in formation of the extra-embryonic vasculature

A major feature of the *Hand1*-null embryos is haemorrhaging of the yolk sac, due to an improperly formed vasculature network (Firulli et al., 1998; Riley et al., 1998). Hand1 has been shown to be required, not for vasculogenesis per se, but for vascular refinement from a primitive to a functional vascular system, including recruitment of smooth muscle cells to the endothelial network (Morikawa and Cserjesi, 2004). Several angiogenic genes were found to be mis-expressed in *Hand1*-null extra-embryonic membranes that may account for the defect, including the signalling molecules vascular endothelial growth factor (*Vegf*), angiopoietin 1 (*Ang1*) and the ephrin B ligand *Efnb2*, implicating Hand1 in their regulation (Morikawa and Cserjesi, 2004). The Notch pathway was also implicated by this study, with *Notch1/4* genes being enhanced in *Hand1*-null yolk sacs together with up-regulation of the downstream gene *Hey1*, which may be an effect of enhanced *Vegf* expression or direct regulation by Hand1. Additionally, *Hand2* is up-regulated in *Hand1*-null yolk sacs, which in this lineage is consistent with a presumptive role of Hand1 repression of Hand2 (Bounpheng et al., 2000).

1.2.10 Hand1 function in trophoblast differentiation

Over-expression studies performed in mice and in the Rcho-1 trophoblast stem cell line revealed that Hand1 can promote trophoblast differentiation and arrest cell growth (Cross et al., 1995)(see section 1.2.6.1). In addition, endogenous *Hand1* expression increases in Rcho-1 cells as giant cells begin to differentiate (Cross et al., 1995).

Moreover, *Hand1*-null mutants arrest at E7.5 with placental and extra-embryonic defects (see section 1.2.6.2), including a block in trophoblast giant cell differentiation resulting in a decrease in giant cell number (Riley et al., 1998). A further study has been performed in trophoblast stem (TS) cells, a model system with which to study trophoblast differentiation and trophoblast invasion *in vitro* (Hemberger et al., 2004). *Hand1*-null TS cells exhibit reduced levels of giant cell differentiation and are around 50% less invasive (Hemberger et al., 2004). In combination, these studies suggest that Hand1 mediates terminal differentiation in the trophoblast lineage via exit from the cell cycle, and support the observation that Hand1 plays a role in cell migration/invasion (see section 1.2.6.7).

1.2.11 Hand1 in dorso-ventral patterning

Hand1 is expressed predominantly at the ventral surface of the anterior and posterior portions of the linear heart tube and subsequently, during and after cardiac looping, *Hand1* is expressed weakly in the right ventricle and at the outer curvature of the left ventricle but not in the inner curvature (Cserjesi et al., 1995; Srivastava et al., 1995). Furthermore, *Hand1*-null cells are excluded from the outer curvature of the left ventricle (Riley et al., 2000). Given these observations, a putative role for Hand1 was suggested in determining dorso-ventral (DV) identity in the developing heart (Christoffels et al., 2000). The study by Togi and co-workers (2004) aimed to delineate the role of Hand1 in DV patterning by mis-expressing Hand1 throughout the ventricular myocardium, but did not really yield any insight into this particular function other than Hand1 appears to act cooperatively with additional transcription factors expressed at the outer curvature of the ventricle. A precise role for Hand1 in establishing DV identity in the heart, therefore, remains elusive.

It has also been suggested that Hand1 is involved in DV patterning in the developing limb, again correlated with the predominant expression of *Hand1* in ventral regions of the limb bud (Fernandez-Teran et al., 2003). However, as with the heart, any definitive role for Hand1 in DV patterning has yet to be determined.

1.2.12 Summary

Hand1 potentially has multiple roles in cardiac morphogenesis, and appears to impact upon multiple genetic pathways. Previous studies of Hand1 have indicated that it may

play a role in cardiomyocyte and neural crest migration, specifically during cardiac looping and outflow tract specification (Riley et al., 2000). Some insights into Hand1 function have come from the collective studies described above that confirm a role for Hand1 in the morphogenesis developing heart and suggest a role for the Hand proteins in cardiomyocyte differentiation, but still the exact functions of Hand1 remain unknown.

Conventional knockout strategies are unsuitable for studying the precise function of Hand1 in the heart *in vivo* because homozygous-mutant embryos die too early from extra-embryonic defects (Riley et al., 1998). In addition, the tetraploid rescued *Hand1*-null mutant embryos are very rare, difficult to generate and die at stages precluding detailed analysis of Hand1 function in the heart, most notably in terms of chamber maturation and outflow tract specification. It seems, therefore, that to understand the precise molecular and cellular role of Hand1 at multiple stages of cardiac morphogenesis, a spatial and temporal targeting strategy is required.

However, in mouse knock-out models where early embryonic lethality precludes detailed investigation of function in the embryo proper, ES cells can be used as an *in vitro* model system in which to study cell specification and differentiation. Furthermore, ES cells are amenable to genetic manipulation in order to study associated gene functions. Therefore, since *Hand1*-null embryos die early from extra-embryonic defects, precluding analysis of Hand1 in the developing heart, ES cells are a particularly useful model in which to study the role of Hand1 in cardiomyocyte differentiation *in vitro*. *Hand1*-null ES cells have been generated previously that differentiated into beating cardiomyocytes and expressed cardiac specific transcripts, indicating that Hand1 was not absolutely required for cardiomyocyte differentiation (Riley et al., 2000). Differences between wildtype and *Hand1*-null ES cells have been noted (unpublished observations), and these form the basis for the studies discussed in Chapter 6 investigating the function of Hand1 in cardiomyocyte differentiation.

1.3 Cardiomyocyte differentiation *in vitro*

1.3.1 ES cells as a model for differentiation

Mouse embryonic stem (ES) cell lines are permanent cell lines that have been derived from the inner cell mass of developing blastocysts. When maintained in the undifferentiated state by culturing in the presence of leukaemia inhibitory factor (LIF), ES cells are able to undergo unlimited self-renewal. Removal of LIF induces spontaneous differentiation. When cultured in suspension, differentiating ES cells will cluster and form 3D aggregates known as embryoid bodies (Figure 1.14).

Embryoid bodies develop into many different specialised cell types derived from the three germ layers of the developing embryo, endoderm, ectoderm and mesoderm. The differentiated cell types include cardiomyocytes, skeletal muscle, blood cells and neuronal cells. ES cells can be directed down particular differentiation pathways by treating with various differentiation factors for example dimethylsulfoxide (DMSO) to induce skeletal and cardiac muscle, and retinoic acid (RA) to induce neuronal development.

Numerous genes exhibit precisely controlled, temporal expression patterns throughout differentiation of embryoid bodies that are comparable to those observed in the developing embryo, which suggests that the pathways controlling differentiation in embryoid bodies *in vitro* reflect those occurring in the mouse embryo *in vivo* (Maltsev et al., 1993).

Recently, there has been great interest in mechanisms governing pluripotency and lineage commitments, to both provide insight into these complicated cellular processes *in vivo*, and also to exploit their potential use in cell-based therapies. It is hoped that ES cell derived cardiomyocytes can be used for cell transplantation therapies to increase cardiac function in patients who have suffered myocardial loss through infarction (Weissberg and Qasim, 2005). Enormous efforts are, therefore, being made to understand the commitment of cells towards cardiomyocytes, and the differentiation of specialised cell sub-types. Yet, despite the many varied studies on ES cell commitment to the cardiac lineage, very little is known about the regulation of cardiac fate other than it most likely involves multiple different signalling pathways.

1.3.2 ES cell differentiation into cardiomyocytes

Spontaneously beating cardiomyocytes usually begin to appear at around day 8 of differentiation, indicating that the appropriate developmental signals for cardiomyocyte differentiation are present. Moreover, it has been demonstrated that differentiation of cardiomyocytes in floating embryoid bodies *in vitro* is comparable to differentiation of foetal cardiomyocytes *in vivo* (Doevendans et al., 2000). Cardiomyocyte differentiation *in vitro* has been characterised on a molecular basis in terms of gene and protein expression profiles, and also physiologically using the patch clamp technique (Figure 1.14).

Cardiac-specific genes and proteins are expressed in a developmentally controlled manner in cardiomyocytes derived from ES cells *in vitro* (Maltsev et al., 1993). Early cardiac genes such as *Nkx2.5* and *GATA-4* are expressed before genes associated with cardiomyocyte differentiation including *ANF*, *Mlc2v*, α-myosin heavy chain (*α-MHC*) and *β-MHC* (Boheler et al., 2002). However, a study in the HM1 ES cell line suggests that cardiomyocytes that differentiate *in vitro* are not mature cardiomyocytes, but more closely resemble those of the early primary myocardium of the embryonic heart tube and regions that become outflow tract, inflow tract and atrioventricular canal (Fijnvandraat et al., 2003a; Fijnvandraat et al., 2003b). This assumption was based upon relatively low levels of expression of *ANF* and *Cx40*, genes that in the developing embryo are associated with the more differentiated working myocardium (see section 1.1.3.5). Although the results of this study must be taken into consideration, they only portray the situation in one ES cell line, and different cell lines are known to show variation in behaviour and gene expression pattern.

Physiologically, ES cell-derived terminally differentiated cardiomyocytes have been shown to have action potentials characteristic of the specialised cells of the atrium, ventricle, sinus node (pacemaker-like) or Purkinjie cells whilst cardiomyocytes at early stages of differentiation exhibit only pacemaker-like action potentials that are typical of primary myocardium (Maltsev et al., 1993; Wobus and Guan, 1998; Boheler et al., 2002). An intermediate stage of cardiomyocyte differentiation is also delineated by action potential, which is thought to be representative of developmentally regulated transitions between different cell types (Maltsev et al., 1993). Again however, the study in the HM1 ES cell line indicated that the electrophysiological characteristics of

cardiomyocytes derived *in vitro* most closely resembled those of the embryonic outflow tract, deemed primary myocardium (Fijnvandraat et al., 2003a).

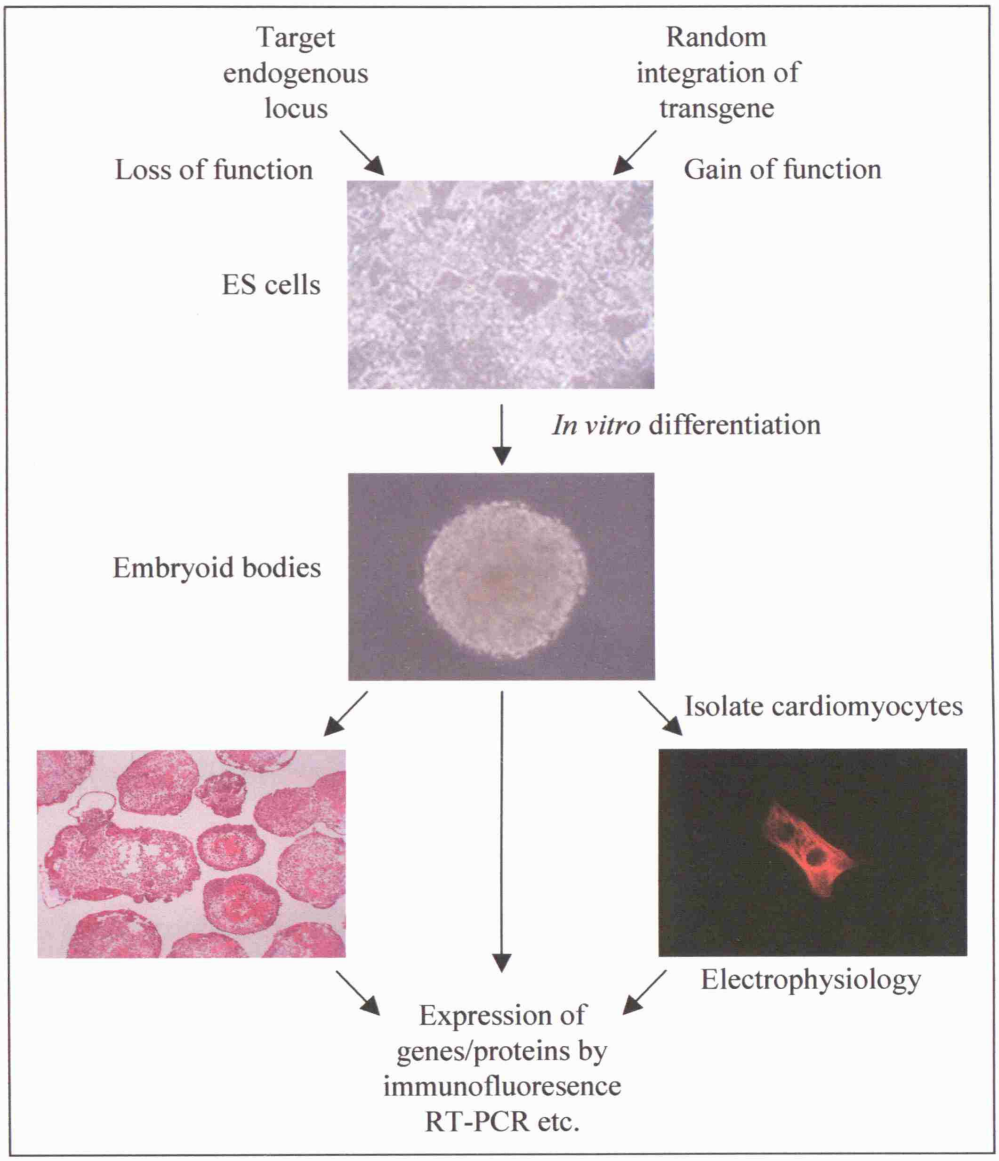


Figure 1.14. ES cells as a model to study cardiomyocyte differentiation. ES cells can be genetically manipulated in order to study loss and gain of function. When cultured in suspension in the absence of LIF, clonal cells proliferate and form 3D aggregates called embryoid bodies and spontaneously differentiate into all embryonic cell types including cardiomyocytes. Embryoid bodies can be studied directly or cardiomyocytes can be isolated. A variety of techniques can be employed to study embryoid body differentiation including electrophysiology, immunofluorescence and RT-PCR.

Since ES cells are able to differentiate into the many different specialised cardiomyocytes in a temporally controlled fashion, they are an excellent model in which to study, at least the early stages of, cardiomyocyte specification and differentiation *in vitro*.

1.3.3 ES cells as a model to study gene function during differentiation

Further to being a good model for differentiation, ES cells can be manipulated genetically by either gene targeting events or by random insertion of a transgene (Figure 1.14). This enables the study of both loss and gain of gene function, the effects of which can be observed in a cell autonomous fashion *in vitro*, in isolation from secondary effects associated with mutant embryo development. *Srf*-null embryos arrest at the onset of gastrulation and lack detectable mesoderm, but the role of SRF was not clear from this early phenotype (Weinhold et al., 2000). *Srf*-null ES cells were, therefore, generated to examine the role of SRF in mesoderm differentiation, and it was demonstrated that SRF was required non-cell autonomously for mesodermal gene expression (Weinhold et al., 2000).

Because of the advantages described above, and the lack of a permanent cell line in which to study cardiomyocyte differentiation, ES cells have been employed to study various aspects of cardiomyocyte lineage specification and differentiation, including the role of transcription factors, signalling molecules and phosphorylation, extra-cellular matrix molecules, and components involved in calcium homeostasis (reviewed in (Boheler et al., 2002).

However, despite the advantages, ES cells are not without their limitations. Different wildtype ES cell lines can behave differently, the reported percentage of ES cells differentiating into cardiomyocytes can vary, for example, from less than 3% (Boheler et al., 2002) to 41% (Wobus et al., 1997). It has also been reported that some ES cell lines can silence the expression of ectopically expressed transgenes hindering analysis of genetically modified lines (Boheler et al., 2002).

1.3.4 Transcriptional control of commitment to the cardiomyocyte lineage and cardiomyocyte differentiation

Several signalling molecules have been implicated in cardiomyocyte differentiation *in vivo* including Wnts, BMPs and FGFs, through their ability to induce cardiac specific transcription factors such as Nkx2.5 and GATA4. However, the exact signals that mediate cardiomyocyte differentiation remain unknown.

ES cells can be cultured with different growth factors/signalling molecules to study their effect on cardiomyocyte differentiation. Treatment of ES cells with Noggin (BMP inhibitor)(Yuasa et al., 2005), TGF-β2 (Kumar and Sun, 2005) or Wnt11 (Terami et al., 2004), for example, results in a marked increase in the number of differentiated beating cardiomyocytes, and an up-regulation of cardiac specific transcription factors. Equally, transcription factors and signalling molecules implicated in cardiomyocyte differentiation can be examined by gene targeting. *GATA4*-null and *Fgfr1*-null ES cell lines differentiate into cardiomyocytes, but at a greatly reduced frequency (Narita et al., 1997; Dell'Era et al., 2003). Studying cell lines in which genes are over-expressed or 'knocked-out' may provide some insight into the processes governing cardiomyocyte differentiation.

1.3.5 Summary

ES cells are a useful model in which to examine the effects of both loss, and over-expression, of *Hand1* on cardiomyocyte differentiation *in vitro*. ES cell studies described in Chapter 6 were, therefore, designed to compliment the principle objective of this PhD: to make use of the tetracycline inducible system to generate a temporally and spatially controlled tet-inducible model with which to study both loss and gain of Hand1 function in the developing heart *in vivo*, with the ultimate goal of underpinning a role for Hand1 in the developing mouse heart. Described in the following section is the background to the Tet system in the context of other available inducible systems, and how this can be applied to establish inducible knock-out of over-expression models for Hand1.

1.4 Tet inducible knock-out of *Hand1*

1.4.1 Introduction

The objective of this project was to generate an inducible knock-out of *Hand1* to study its function in the developing heart. It was decided to create a tet-inducible knock-out of *Hand1* – placing the Tet-Off system into the endogenous *Hand1* locus. Previous use of the tet-inducible systems *in vivo* has been predominantly in the context of transgene over-expression, and has been problematic, for example, random integration, low or mosaic expression of activators and leaky expression of the responder genes in the absence of transactivation (Rossant and McMahon, 1999), although transcriptional silencers have been engineered to reduce basal levels of leaky expression from responder genes (Zhu et al., 2001). Nevertheless, the tetracycline controlled gene expression systems have been used successfully for a wide range of studies into gene function, and there exists a relatively large collection of a number of transgenic and three knock-in tetracycline controlled mouse strains (http://www.zmg.uni-mainz.de/tetmouse/).

The knocking-in of Tet-On/Off regulatory sequences and Tre2 response sequences into an endogenous locus to study gene function is a model system, first described in Nature (Shin et al., 1999), and ensures that the gene of interest will be regulated in a spatially appropriate manner, and a similar approach has been used successfully in three other studies since (Holland et al., 2002; Taylor et al., 2003). Using this system, doxycycline would be administered at various stages of development to 'switch off' *Hand1* expression and the effects on the developing heart observed. Additionally, the Tet-Off system can be exploited to study the effects of Hand1 over-expression throughout development. The main focus, however, will be from E8.5 during cardiac looping and E9.5 during both cardiac neural crest migration and anterior heart field contribution to the outflow tract.

1.4.2 The Tet-Off system

In *E. coli*, the Tet repressor protein (TetR) binds to the tet operator sequences (*tetO*) of the tetracycline-resistance operon on the Tn*10* transposon, in the absence of tetracycline

(Tc) (Gossen and Bujard, 1992) or its derivatives such as doxycycline (dox). This blocks transcription of the genes on the operon. The TetR and *tetO* form the foundations of the Tet-Off and Tet-On mammalian expression systems.

The Tet-Off System has two components (Figure 1.15A). Therefore, for use as a model system for studying gene function, it requires the generation of two separate mouse strains. The first component is the transactivator (tTA), which is based on the TetR. The tTA is a fusion protein, comprising of the first 207 amino acids of the TetR at the N-terminus, and the last 127 amino acids of the Herpes simplex virus virion protein 16 (VP16) activation domain at the C-terminus. The addition of the VP16 activation domain transforms TetR from a transcriptional repressor into a transcriptional activator – the tTA. The Tet-On transactivator (rtTA) is founded on a 'reverse' Tet repressor (rTetR) and differs from the tTA by four amino acids.

The second component is the tetracycline-response element (TRE) placed upstream of a gene of interest. The TRE is composed of seven consecutive repeats of the 42-base pair (bp) *tetO* sequence/motif. These repeats are just upstream of the minimal cytomegalovirus (CMV) promoter ($P_{min\ CMV}$), which lacks the strong enhancer elements that are part of the complete CMV immediate early promoter. Since the enhancer elements are missing, the TRE is completely silent, until the tTA (or rtTA) binds and induces expression of the downstream gene (Figure 1.15B).

Gene expression in the Tet-Off system can be controlled with tetracycline (Tc) or its functional analogue doxycycline (dox)(Figure 1.15C). Under normal conditions i.e. in the absence of dox, gene expression is 'on' as the tTA is able to bind the TRE and induce gene expression. Addition of dox switches gene expression 'off' - dox binds to, and induces a conformational change in the tTA so that it is unable to bind the TRE and, therefore, also unable to transactivate the downstream gene. With the Tet-On system, addition of doxycycline has the opposite effect on the rtTA. The rtTA binds the TRE and activates transcription in the presence of dox, removal of dox, therefore, switches 'off' gene expression.

Dox can be administered to embryos via pregnant mothers in food, drinking water, or by injection since dox is able to cross the placenta without affecting embryonic development. Dox is administered at non-pathological levels ensuring it is not

detrimental to mammalian cells, and so there are no adverse effects except in cases of long-term administration (see below; (Eger et al., 2004).

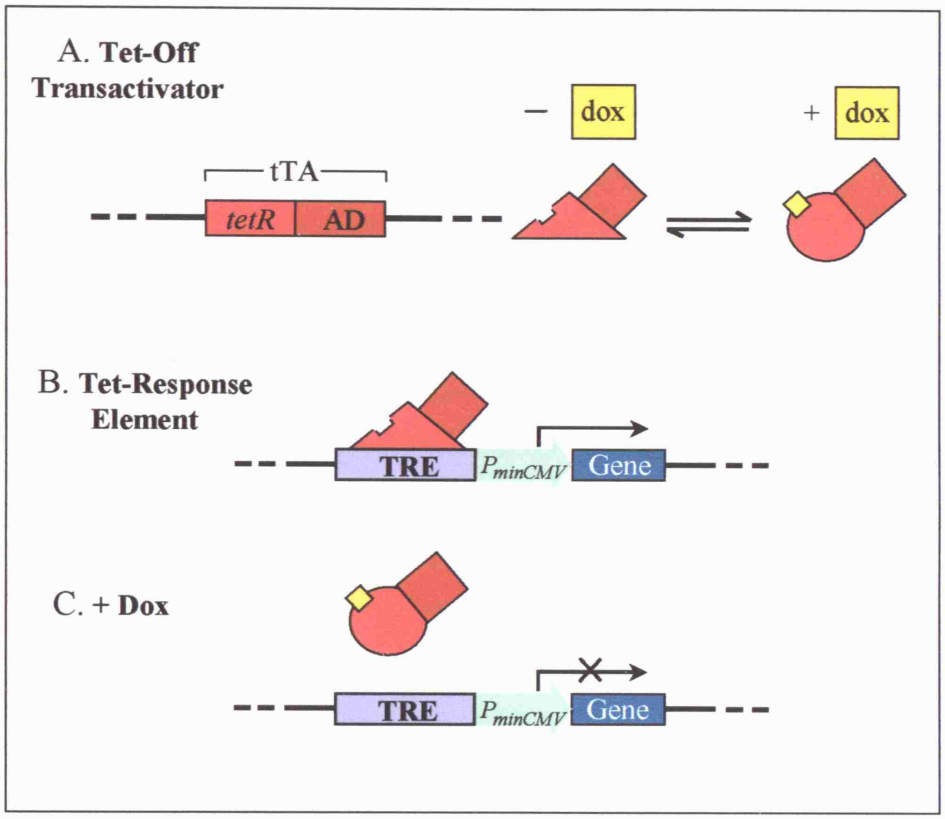


Figure 1.15. The Tet-Off system. (Adapted from Tet Products – an Overview, CLONTECHniques July 2000, Clontech). The Tet-Off system has two components, the Tet-Off transactivator (A; tTA), which is a fusion protein consisting of the *tet*R repressor DNA binding domain fused to the VP16 activation domain (AD), and (B) the Tet-response element (TRE) upstream of a minimal CMV promoter (P_{minCMV}) and a gene of interest (Gene). In the absence of dox, the tTA can regonise and bind to the TRE inducing expression of the downstream gene (B). Dox induces a conformational change in the tTA (A) inhibiting it from binding the TRE and preventing, or 'switching off', expression of the downstream gene (C).

1.4.3 Tet-Off compared to Tet-On

The Tet-Off system is reportedly 'quicker' than the Tet-On system in terms of response to dox. With Tet-On, expression is 'switched on' requiring transcription and translation of the gene/protein in question. Plus, 'switching off' gene expression in the Tet-On system, requires that all traces of dox are removed from the embryonic circulation for

complete inhibition which takes longer than 'switching off' expression using the Tet-Off system. For purposes of 'switching off' *Hand1* expression, the more rapid Tet-Off system seemed the most appropriate approach. Additionally, there is potential for 'leaky' expression i.e. expression of the gene of interest in the absence of transactivation, in the 'off' situation using the Tet-On system, whereas with Tet-Off, expression occurs in the absence of dox. With the Tet-On system, if the response element is leaky in the absence of transactivator/dox, or if there is still weak recognition of Tre2 sequences by the Tet-On transactivator even in the absence of dox, the result is inappropriate expression from the response element (see references within (Zhu et al., 2002). To combat this problem, a transcriptional silencer (tTS) has been engineered that is a fusion of TetR with the KRAB-AB transcriptional repressor domain of the Kid-1 protein, that binds to the TRE only in the absence of dox and inhibits expression of the gene of interest *in vitro* (Freundlieb et al., 1999) and *in vivo* (Zhu et al., 2002), thus abrogating the leaky responder gene expression. As with the Tet-Off transactivator, dox induces a conformational change in the tTS resulting in dissociation from the TRE. This relieves the repressive effect enabling the Tet-On transactivator to bind and induce expression of the downstream gene. Unfortunately, given that the tTS works on the same principles as the tTA (Tet-Off transactivator), the two cannot be used in conjunction with each other, the tTS can only be used with Tet-On system approaches.

Tet-On also requires that the mice be maintained on doxycycline in order to maintain gene expression, this incurs potential problems in maintaining an appropriate long term dox dosage, as opposed to administration of dox at a single 'switching off' timepoint. Additionally, the antibiotic activity of dox can have adverse effects on the intestinal flora of mice, causing diarrhoea and in a few animals, colic (Eger et al., 2004), making prolonged exposure to dox, necessary with the Tet-On system, undesirable.

1.4.4 Advantages of tet-inducible knock-out versus other knock-out strategies

1.4.4.1 Inducible systems

Other inducible systems have been experimented with in eukaryotic cells in order to achieve spatial and temporal gene regulation, such as metal ions, heat shock and hormones (Gossen and Bujard, 1992). However, these systems suffer from 'leakiness'

in the inactive state. Another downfall of some of these systems is the pleiotropic effects of the inducing factor such as elevated temperature or high glucocorticoid hormone action (Lee et al., 1988). Lac repressor/operon systems have also been tested in mammalian cells but tend to be hindered by the slow and inefficient action of the inducer isopropyl β-D-thio-galactopyranoside (IPTG) (Gossen and Bujard, 1992). In the case of the tet-inducible system, the tTA is unable to bind the *tetO* sequences in the presence of dox or Tc and the TRE is virtually silent, so the system is less likely to suffer from leakiness. Unlike other inducers, dox is not harmful, as described above, and therefore, makes the tet-inducible system preferable. Additionally, the system does not suffer from slow, inefficient action as the TetR binds Tc/dox extremely tightly and efficiently, and it is relatively rapid in effect.

1.4.4.2 Tet-Off versus Cre-*lox* for generating a conditional knock-out

An alternative strategy for gaining insight into gene function in a specific lineage or organ such as the heart, is the Cre-*lox* strategy. This approach makes use of the site-specific P1 bacteriophage Cre recombinase. Cre recombinase recognises 34 base pair *loxP* sequences and catalyses recombination between a pair of uniformly orientated sites. When the *loxP* sites are arranged in cis and same orientation, excision by Cre and subsequent recombination between the sites splices out the intervening sequence.

Cre recombinase can be put under the control of a tissue-specific promoter or targeted to a specific locus providing tissue-specific expression of Cre. When the Cre recombinase strain is crossed with a strain that carries a floxed allele (sequence flanked by *loxP* sites) the floxed sequence is knocked-out, and the gene of interest therefore inactivated, only in tissues expressing cre recombinase.

If a tissue-specific promoter, which may lack distal temporal and spatial regulatory elements, is used to drive Cre recombinase expression, the levels of recombinase produced may not be sufficient in every cell of the tissue in question to completely excise the target gene (Nagy, 2000). This would result in partial excision that would give a mosaic phenotype that is difficult to analyse and interpret correctly.

It can also be difficult to study quickly changing, dynamic events with Cre recombinase because it takes time to complete the process between onset of recombinase expression

to knocking-out the floxed sequence. Loss of function of the target gene is gradual rather than immediate (Nagy, 2000).

The Tet-Off system incorporates both 'on' and 'off' situations of the gene in question. Specific time points can be selected, and the system provides the choice of inducing or repressing expression. With cre-*lox* conditional knock-out strategies, the gene is knocked-out when Cre recombinase is expressed, and therefore, only provides a single time point (limited by the promoter driving the Cre), and is restricted to an 'off' situation. The Tet-Off system also provides the possibility of studying effects of the knock-out in more than one lineage unlike the tissue-specific control of Cre recombinase, for which such approaches would require multiple mouse strains. Additionally, with the Tet systems there is scope for potentially rescuing a phenotype at a later stage in development, or post-natally by switching the gene on again. Furthermore, it may be possible to titrate the levels of dox administered in order to produce hypomorphic levels of expression of the gene in question in that the tTA can be maintained in a partially activated state with low levels of dox/Tc. Attempts are being made to design temporally controlled tissue-specific Cre expression systems. One example uses tamoxifen as the inducing ligand and Cre fused to a mutated ligand-binding domain of the oestrogen receptor that is incapable of binding endogenous oestrogen (Brocard et al., 1997). Instead, the Cre fusion binds the oestrogen antagonist tamoxifen, inducing translocation to the nucleus where Cre can execute its function. However, so far this system appears to suffer from incomplete activity and only partial excision in a subset of the targeted cell type (Danielian et al., 1998). Hayashi and McMahon (2002) developed a mouse line that constitutively expressed a Cre-mutated oestrogen receptor fusion, but this was also seen to effect only partial excision. Additionally, the levels of tamoxifen required for Cre excision were toxic to the embryos (Hayashi and McMahon, 2002).

1.4.5 Summary

The Tet-Off system was chosen for engineering an inducible knock-out of *Hand1* because it allows both temporal and spatial regulation of the gene at a wide range of timepoints throughout development. This is particularly useful for determining the role of Hand1 in the heart since from its expression pattern and the limited results from gene targeting, tetraploid rescue and chimera studies, Hand1 appears to have multiple roles in

the developing heart at different stages. A tet-inducible-*Hand1* model system offers another major advantage. In addition to loss of function studies, employment of the Tet-Off system has enabled the investigation of *Hand1* over-expression in the developing embryo. Furthermore, it includes the potential for examining other embryonic tissues where *Hand1* is also expressed without the necessity of having multiple mouse strains, since one strain is required for the Tet-Off transactivator, and one for the tet-inducible responder.

1.5 Principal aims of these PhD studies

Vertebrate heart development involves complex interplay between many different molecular and morphogenetic events, yet how the molecular systems translate into the morphogenetic processes that occur during normal cardiogenesis is not fully understood. Hand1 is a transcription factor that has been implicated in various stages of cardiac morphogenesis. However, *Hand1*-null embryos die early due to extra-embryonic defects, precluding precise analysis of Hand1 function in the developing heart. Therefore, the objective of this project was to create a temporal and spatial knock-out of *Hand1* using the Tet-Off system. This was to be achieved by generating two mouse strains, one placing the Tet-Off transactivator under the control of the endogenous *Hand1* locus (*Tet-Off-Hand1*), which in turn would induce expression of *Hand1* in the second strain via a tet-response element (TRE) (*Tre2-Hand1-EGFP*). Embryonic development would proceed normally as the tet-responsive *Hand1* would be expressed solely in the areas of the developing embryo where endogenous *Hand1* is normally expressed, hopefully by passing the extra-embryonic complications. Addition of doxycycline at any chosen time point would inhibit the Tet-Off transactivator and thus switch off *Hand1* expression, creating a temporal knock-out, with the aim of underpinning a role for Hand1 in the developing heart.

Additionally, since *Hand1*-null embryos die early from extra-embryonic defects, precluding analysis of Hand1 in the developing heart, ES cells are a particularly useful tool for studying the function of Hand1 in cardiomyocyte differentiation *in vitro*. Therefore, it was decided to examine the effect of Hand1 on cardiomyocyte differentiation further by generating ES cell lines that precociously and over-express *Hand1*, and to compare these to the wildtype and *Hand1*-null ES cell lines. Not only is this study interesting in it's own right, but also significantly compliments the *in vivo* tetracycline-inducible knock-out and over-expression studies.

The second chapter describes the cloning of the constructs and the subsequent efforts made to generate various mouse strains. The transactivator strain, *Tet-Off-Hand1*, was generated successfully. However targeting the responder construct, *Tre2-Hand1-EGFP*, failed at the point of germline transmission. A transgenic approach was then adopted for the responder as it offered many advantages, such as pre-selection of a variety of lines with different copy number. Germline transmission was obtained for a total of

four independent *Tre2-Hand1* lines, although two were received only recently and have not been tested further.

The third chapter provides details of the extensive *in vitro* testing performed on the three constructs that demonstrated that the system was working appropriately in terms of expression, repression by doxycycline and transcriptional function.

The fourth chapter describes the characterisation of the mouse strains generated and the progress made so far towards successfully generating the temporal and spatial knock-out of *Hand1*. The expression pattern of the Tet-Off transactivator was investigated in the *Tet-Off-Hand1* strain, and was shown to reflect that of wildtype *Hand1* in developing embryos. One of the *Tre2-Hand1* responder lines failed to express transgenic *Hand1*. A second line did express the *Hand1* transgene, but only at a low level. A third responder line, *Tet-Hand1*, was obtained from collaborators (T. Mohun, National Institute of Medical Research (NIMR), Mill Hill, London). *Tet-Hand1* is known to express at a relatively high level in neo-natal mice. Therefore, efforts to obtain the temporal knock-out of *Hand1* are ongoing with this third *Tet-Hand1* line.

One significant advantage of utilising a transgenic responder strain was that it creates a bimodal model with which to study Hand1 function. It enables not only a loss of function to be established via the temporal knock-out, but also allows analysis of gain of Hand1 function. Embryos can be generated that over-express *Hand1*. Crucially, *Hand1* is over-expressed exclusively in *Hand1*-expressing regions due to the Tet-Off transactivator being targeted to the endogenous *Hand1* locus. The *Hand1* over-expression phenotype and analysis of potential causative mechanisms are discussed in chapter five.

Chapter six tells of a study undertaken in ES cells, which was designed to compliment the *in vivo* work described above. ES cell lines were created that precociously, and subsequently over-express *Hand1* throughout differentiation. The over-expression cell lines were compared to wildtype and pre-existing *Hand1*-null ES cells lines to examine the effects of Hand1 on cardiomyocyte differentiation *in vitro*.

The final chapter describes ongoing studies and areas of potential future work using both the *in vivo* and *in vitro* models created during this PhD.

Despite many efforts to understand the function of Hand1 in cardiac morphogenesis, and the suggestion that it may be involved in processes such as outflow tract formation, left ventricle specification and cardiac neural crest migration, its exact role remains

unclear. It is hoped that the *in vivo* and *in vitro* studies described within will shed some light on the precise role that Hand1 plays in the developing heart, during cardiac morphogenesis and in cardiomyocyte differentiation, and that it will also provide further insight into how anomalies in cardiac morphogenesis may underlie congenital heart disease.

Chapter 2: Construct and mouse strain generation

2.1 Introduction

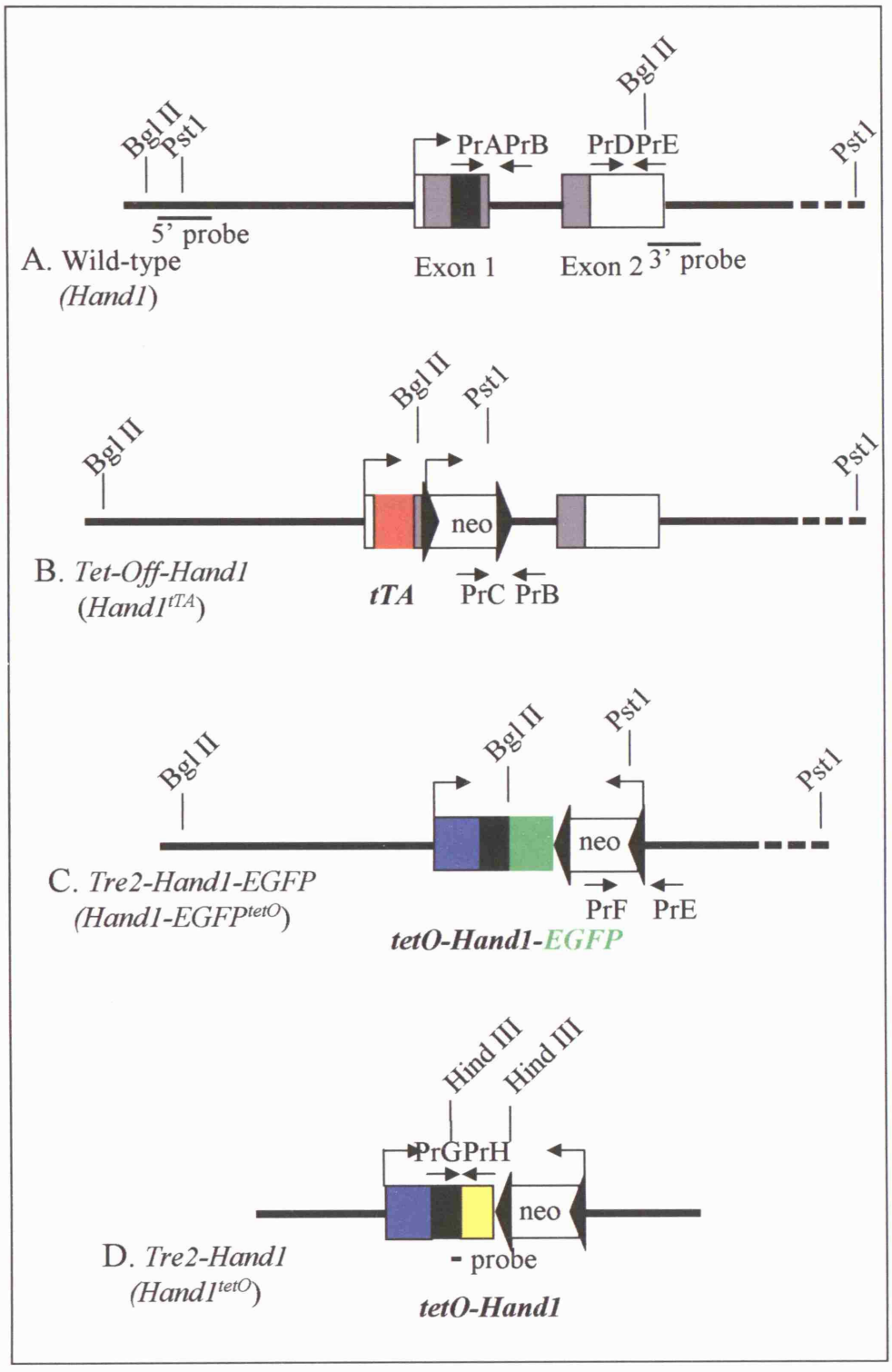
2.1.1 Incorporating Tet-Off system into *Hand1* locus

The Tet-Off system has two components, the Tet-Off transactivator and the tet response element (see section 1.4.2). Therefore, to create a mouse model using the Tet-Off system, two independent mouse strains are required. The Tet transactivator (*Tet-Off-Hand1*; allele $Hand1^{tTA}$) and Tet response element (*Tre2-Hand1-EGFP*; allele $Hand1^{tetO}$) constructs (Figure 2.1B and 2.1C respectively) were cloned based on two *Hand1* targeting vectors used for the original *Hand1* gene targeting (Riley et al., 1998; Riley et al., 2000). *Tet-Off-Hand1* places the transactivator under the control of the full length endogenous *Hand1* promoter, plus upstream enhancer elements, ensuring the transactivator is subject to the same spatial and temporal regulation as *Hand1*. The transactivator in turn binds the Tet-response element in the *Tre2-Hand1-EGFP* construct, driving expression of *Hand1* and *EGFP*. EGFP (Enhanced Green Fluorescent Protein) was included in the response element construct as a marker for *Hand1* expression in transheterozygote ($Hand1^{tTA}$/ $Hand1^{tetO}$) embryos. The original strategy of targeting the response element and *Hand1-EGFP* to the *Hand1* locus was to ensure the construct integrated in an appropriate chromatin state, and in an active locus, to allow appropriate expression of the *Hand1-EGFP* fusion.

Initially, both constructs were targeted separately to the endogenous *Hand1* locus by homologous recombination. Germline transmission was achieved for the *Tet-Off-Hand1* strain. However, because germline transmission was not obtained for the *Tre2-Hand1-EGFP* construct, an alternative transgenic approach was adopted for the responder, for which the *Tre2-Hand1* construct was cloned (Figure 2.1D). The most significant advantage of using a transgenic responder was that it generated a bimodal model with which to study Hand1 function in the developing heart. There is the potential to both knock-out *Hand1*, in a temporal and spatial manner, and also to over-express *Hand1* exclusively in regions of the heart where *Hand1* is expressed.

Figure 2.1. Incorporating the Tet-Off system into the *Hand1* locus. Map of the *Hand1* locus, the *Hand1*^{tTA} and *Hand1-EGFP*^{tetO} targeting vectors, and the *Hand1*^{tetO} transgenic vector. (A) The wildtype *Hand1* gene consists of two exons (boxes: non-coding regions indicated in white, coding region is shaded, bHLH domain is shown in black) separated by a 1.5 kb intron. The targeted *Hand1* locus contains (B) the transactivator (tTA, shown in red) under the control of the endogenous *Hand1* promoter or (C) the tet-responsive *Hand1-EGFP* fusion, the Tre2 is blue, full length Hand1 cDNA is black and EGFP is shown in green; targeting vectors based on the previously described *Hand1*^{lp} and *Hand1*^{neo} vectors respectively (Riley et al 1998; Riley et al 2000). (D) The tet-responsive *Tre2-Hand1* transgenic vector (boxes as (C) plus *β-globin* poly (A) is shown in yellow).

Also shown in A-C are the *Bgl*II and *Pst*I restriction enzyme sites, and the corresponding 5' and 3' probes respectively, used for Southern blotting to identify correctly targeted ES cell clones. Similarly shown in D are the *Hind*III sites and the corresponding probe used to identify transgenic ES cell clones. The primers indicated, PrA-PrH, were used to confirm positive ES cell clones by PCR. Pr, primer. For primer sequences see Appendix 2.


Bgl II
Pst1
PrAPrB
Bgl II
PrDPrE
Pst1
5' probe
A. Wild-type
(Hand1)
Exon 1
Exon 2
3' probe
Bgl II
Bgl II
Pst1
Pst1
neo
B. Tet-Off-Hand1
(Hand1tTA)
tTA
PrC PrB
Bgl II
Bgl II
Pst1
Pst1
neo
C. Tre2-Hand1-EGFP
(Hand1-EGFPtetO)
PrF PrE
tetO-Hand1-EGFP
Hind III
Hind III
PrGPrH
neo
D. Tre2-Hand1
(Hand1tetO)
- probe
tetO-Hand1

2.1.2 Employing a transgenic responder approach to generate a loss of function model for *Hand1*

Transgenics usually involves ectopic over-expression of the transgene. The transactivator has been correctly targeted to the *Hand1* locus and thus will be subject to the temporal and spatial regulation of the endogenous *Hand1* locus. This is the crucial factor for this tet-inducible knock-out of *Hand1* since it ensures that the *Tre2-Hand1* responder is expressed only in *Hand1*-expressing cells, and that there is no ectopic *Hand1* expression despite the use of a transgenic responder.

Targeting the responder to the endogenous locus ensures that the DNA is in the correct chromatin state for appropriate transcription, and also that the site of integration is not silent. However, the responder does not necessarily need to be 'targeted' provided that it can be established that the *Tre2-Hand1* transgene has not integrated into a 'silent' locus and can be activated by a Tet-Off transactivator, which can be tested using the *Tre2-Hand1* ES cell clones generated. In order to test that *Hand1* expression could be induced via the *Tre2-Hand1* transgene in ES cells, it was necessary to use a Tet-Off transactivator construct under the control of a promoter that is active in ES cells, such as the thymidine kinase (TK) promoter (pBig3r; (Strathdee et al., 1999) or the chick β-actin promoter (pCAGtTA)(Kitajima et al., 2002). The *Tet-Off-Hand1* construct was inappropriate for this purpose since it is under the control of the *Hand1* promoter region, which is not active in ES cells.

A potential caveat of the tet-inducible system is inappropriate levels of expression of the gene of interest from the response element. There is an inherent potential to over-express *Hand1* using the tet system. For instance, the VP16 activation domain of the Tet-Off transactivator is a potent activation domain and may induce high levels of transgene expression, or the transgene may become integrated into a locus that is open to abundant transactivation. The transgenic *Tre2-Hand1* mice were generated by electroporation of transgenic ES cells, rather than by pro-nuclear injection, since transgene copy number could be selected for. This is an important advantage of using ES cells for the transgenic approach, as several lines with different copy numbers of transgene can be established, with potentially differing expression levels, although transgene expression will still be dependent upon the site of integration. These lines can

be tested for appropriate levels of *Hand1* expression compared to endogenous *Hand1*, so that transgenic *Hand1* expression can be normalised to endogenous levels.

Additionally, the derivation of transgenic animals provides a higher frequency of obtaining appropriate founder animals as compared to those derived from homologous recombination in ES cells. For all reasons discussed above, a transgenic approach was adopted for the responder strain.

Exploiting the advantages of a transgenic responder does however mean that the *Hand1* locus will not be targeted and both copies of *Hand1* will be present in transgenic *Tre2-Hand1* mice. Whilst this is advantageous for studying *Hand1* over-expression (see below; section 2.1.4), it necessitates the generation of a *Hand1*-null background by other means in order to create the tet-inducible knock-out of *Hand1*. Therefore, the *Tre2-Hand1* transgenic responder was crossed into the $Hand1^{neo}$ background i.e. with the targeted *Hand1* mice available in house (Riley et al., 1998). The heterozygous $Hand1^{neo}$/transgenic *Tre2-Hand1* mouse can then be crossed with the *Tet-Off-Hand1* mouse, resulting in a proportion of one in eight embryos that are compound heterozygote embryos/mice (Figure 4.1). Compound heterozygotes are null for the endogenous *Hand1* locus, but carry the Tet-Off transactivator and the *Tre2-Hand1* transgene ($Hand1^{neo}/Hand1^{tTA}/Hand1^{tetO}$) thus expressing *Hand1* exclusively under the control of the Tet-Off system. Once compound heterozygotes have been generated it becomes possible to evaluate the effect of loss of Hand1 function on the developing heart, using doxycycline to switch off *Hand1* expression at various stages of embryogenesis.

2.1.3 Regulation of *Tre2-Hand1* expression with doxycycline

The tet inducible knock-out of *Hand1* is induced with the tetracycline derivative doxycycline (dox) (Figure 2.2). Administration of dox to pregnant mothers carrying compound heterozygote ($Hand1^{neo}/Hand1^{tTA}/Hand1^{tetO}$) embryos will repress the expression of the transactivator, and switch off *Hand1* in the embryos at a chosen timepoint. Shin *et al.* (1999) found that repression of their particular transactivator was complete six hours post administration. Whilst this provided a possible time scale for the effect of dox on the *Tet-Off-Hand1* transactivator, the *in vivo* kinetics of transactivator expression/repression still had to be determined, by northern blot analysis, specifically for the *Hand1* system.

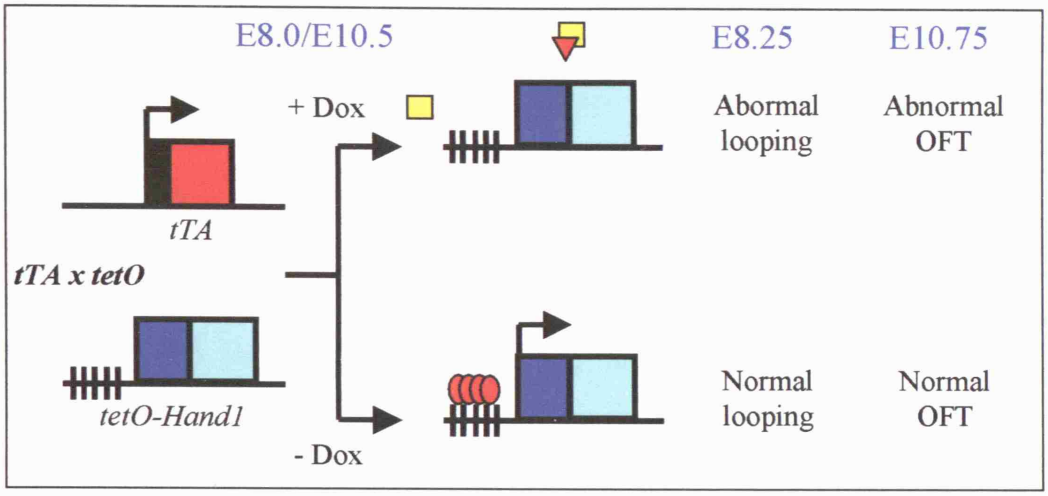


Figure 2.2. The strategy for regulating *Tre2-Hand1* expression by dox. (Adapted from Shin et al., 1999) Dox administration to $Hand1^{tTA}/Hand1^{tetO}$ embryos via the mother will repress the expression of $Hand1^{tetO}$ and this should occur by six hours post dox administration as determined by northern blot analysis (Shin et al., 1999). Therefore, administration of dox to $Hand1^{tTA}/Hand1^{tetO}$ embryos at E8.0 should re-capitulate the unlooped heart phenotype observed in tetraploid-rescued *Hand1*-null embryos. Further manipulation of dox administration to pregnant females at different time points, for example during outflow tract formation, should enable us to repress *Hand1* and dissect out its role in cardiac morphogenesis.

2.1.4 Employing a transgenic responder approach enables gain of function studies

A further advantage of utilising a transgenic approach for the responder strain is that it provides the opportunity not only to carry out loss of function, but also to carry out gain of function analyses to study Hand1 function in the developing heart. Transheterozygote embryos can be generated that carry both the transactivator and the transgenic responder on a heterozygous *Hand1* background (the transactivator is targeted to the *Hand1* locus any animals carrying the transactivator will be heterozygous for *Hand1*; Figure 2.1). If expression levels from the *Tre2-Hand1* transgene are high enough, *Hand1* will be over-expressed exclusively in the areas of the developing heart in which *Hand1* is normally expressed since the transactivator is under control of the *Hand1* locus, and as such represents an entirely novel gain of function approach.

2.1.5 The Targeting constructs

2.1.5.1 Tet-Off-Handl

The Tet-Off transactivator construct (Figure 2.1B) was based on an original *Handl*gene puro targeting vector *Handl*^{lp} described previously (Riley et al., 2000). The Tet-Off transactivator and a rabbit intron/poly(A) (for correct splicing and processing), were incorporated into this puro targeting vector in place of the luciferase gene and puromycin resistance cassette. A neomycin resistance cassette flanked by *lox*P sites (neo^{r}) was also inserted, in the same orientation as the transactivator, for selection of recombinants. The 5' and 3' arms on either end of the construct were retained at 6 kb and 2 kb respectively. The 5' and 3' arms are homologous to the endogenous sequence flanking *Handl* and so act as the sites of homologous recombination resulting in the functional components of the endogenous coding sequence of *Handl* being replaced by the tetracycline transactivator cassette. The 6 kb 5' arm is sequence 5' of the translation start site of *Handl* and contains a fully intact *Handl* promoter ensuring appropriate transcription initiation. The 2.1 kb 3' arm starts at the beginning of the intron, includes the majority of exon 2 and 0.4 kb downstream of the translation stop codon. The targeting into the entire endogenous *Handl* locus ensures that the transactivator is subject to the same temporal and spatial regulation as *Handl*.

2.1.5.2 Tre2-Handl-EGFP

The Tet-response element construct (Figure 2.1C) was based on a *Handl* neo targeting vector *Handl*^{neo} used in the original *Handl* gene targeting studies (Riley et al., 1998). The Tre2 response element upstream of a *Handl-EGFP* fusion was incorporated into the neo targeting vector containing a *lox*P-flanked neomycin resistance cassette in the opposite orientation to *Handl-EGFP*, and 5' and 3' arms for homologous recombination. The arms of the *Tre2-Handl-EGFP* construct differ to those of the *Tet-Off-Handl* construct. The 3' arm is 2.6 kb and starts 0.4 kb downstream of the translation stop codon in exon 2. The 5' arm is 5.7 kb and is lacking 0.3 kb upstream of exon 1. Correct targeting, therefore, disrupts the endogenous *Handl* minimal promoter region. This assures the Tre2 remains silent and *Handl-EGFP* is not expressed until the transactivator binds the response element sequences. In addition, targeting the response element and *Handl-EGFP* to the endogenous locus, ensures that the construct is in an appropriate chromatin state for transactivation.

2.1.5.3 The transgenic responder, Tre2-Hand1

As the transgenic *Tre2-Hand1*construct (Figure 2.1D) was not to be targeted to the endogenous locus, the construct was simply designed with the full length *Hand1* cDNA cloned downstream of the Tre2 and upstream of β-globin poly (A) using the Tre2 vector (Clontech), followed by the insertion of a *lox*P-flanked neomycin resistance cassette in the opposite orientation to *Hand1* for selection of positive clones.

Although it was shown that the EGFP moiety does not affect Hand1 function *in vitro* (see section 3.3.2) and it is a potentially useful marker, a *Hand1-EGFP* fusion was not included in the transgenic construct since EGFP is more stable than Hand1. For future phenotype analysis experiments in compound/transheterozygote embryos, it is crucial that the time frame of 'switching off' of *Hand1* expression is as small as possible. Therefore, the stability of EGFP could affect the response of Hand1 to dox such that the Hand1-EGFP fusion protein may be active for a prolonged period following dox administration.

2.2 Methods

2.2.1 Cloning of targeting vectors

2.2.1.1 Tet-Off-Hand1

An *Eco*R1 + *Bam*H1 1kb fragment of pTet-Off containing the Tet-Off transactivator was ligated into bluescript (pKS$^+$tTA). The 0.8 kb rabbit β-globin II intron and poly(A) was excised from pAPEV (Placental Expression Vector) with a *Sal*1 + *Nco*1 digest, the 5' overhangs were filled in using DNA polymerase 1, Large (Klenow) fragment, and the fragment was ligated into *Spe*1 site of pKS$^+$tTA. The 1.9 kb PGK neomycin resistance cassette flanked by *lox*P sites (PGKneo^r) was added to this intermediate by ligating into *Not*1, as a filled-in *Eco*R1 fragment from the *Hand1*^{neo} targeting vector, downstream of the poly (A). The 4.34 kb insert was then excised and ligated into the *Hand1*^{lp} targeting vector. The intermediate plasmid was linearised with *Acc*651, filled in and the appropriate insert fragment partially digested with *Pvu*II using 20 ng/μl ethidium bromide in the digest reaction mix. The insert was filled in, gel purified and ligated into a filled in *Sal*1 + *Bgl*II digest of *Hand1*^{lp}.

2.2.1.2 Tre2-Hand1-EGFP

Hand1 coding sequence was cloned by PCR from a Flag-Hand1 construct. The 5' primer (primer 392; see Appendix 2) was designed such that it introduced an EcoR1 site in frame with *Hand1*, just upstream of the ATG start codon. The 5' primer also includes the immediate 5' flanking sequence (TCC AAC) as a putative 'kozak' site for ribosome recognition and translation initiation. The *Hand1* coding region PCR product was cloned into the pEGFP-N1 vector (Clontech). Genes that are cloned into the multiple cloning site (MCS) of the pEGFP-N1 vector are expressed as fusions to the N-terminus of EGFP, as long as there are no intervening stop codons and they are in the same reading frame as *EGFP*. The 3' primer (primer 393; see Appendix 2) was therefore designed to remove the *Hand1* TGA stop codon and to be in frame with *EGFP* in the resultant fusion. A *Kpn*1 site was incorporated into the 3' primer for subsequent sub-cloning of the PCR product. The 0.67 kb PCR product was digested with *Eco*R1 and *Kpn*1 and ligated into *Eco*R1 + *Kpn*1 sites of pEGFP-N1. The 0.45 kb *Xho*1 + *Eco*R1 TRE2 fragment was excised from pTRE2 and ligated into *Eco*R1 and *Xho*1 upstream of the *Hand1-EGFP* fusion. The *Tre2-Hand1-EGFP* was excised with a *Xho*1 and *Afl*II digest, filled in and ligated into the *Bam*H1 site of the $Hand1^{neo}$ targeting vector.

2.2.1.3 Tre2-Hand1

Full length *Hand1* cDNA was ligated into *Bam*H1 + *Eco*RV sites of pTRE2, downstream of the TRE, as a *Bam*H1/filled-in *Kpn*1 fragment from pKS^{+}Hand1. The $PGKneo^{r}$ cassette, flanked by *lox*P sites, was inserted as a filled-in *Eco*R1 fragment from the $Hand1^{neo}$ targeting vector, into *Sap*1 of pTre2-Hand1, downstream of *Hand1* and the β-globin poly (A), and in the opposite orientation to *Hand1*. The $PGKneo^{r}$ cassette contains strong promoter regions of prokaryotic origin that can affect endogenous gene function when introduced into mammalian cells. It is, therefore, preferable to insert the $PGKneo^{r}$ cassette in the opposite orientation to reduce the possibility of such interference occurring.

2.2.2 Generation of an inducible knock-out of *Hand1*

The three constructs were sent off to a commercial gene targeting company (genOway, France) for generation of an inducible knock-out of *Hand1*. Each project was divided into three main phases:

- Electroporation of the targeting vector into embryonic stem (ES) cells.
- Recombinant ES cell injections into blastocysts.
- Chimera breeding: Generation of heterozygous mice.

2.2.3 genOway ES cell electroporation

The constructs were targeted separately by electroporation in ES cells.

genOway checked the plasmids had not degraded during shipment by running an aliquot on an agarose gel, prior to amplifying and purifying the plasmids using a standard Qiagen maxiprep method and diagnostic restriction digest. The plasmids for the targeting constructs were then linearised: pTet-Off-Hand1 with *Kpn* I, pTre2-Hand1-EGFP with *Sal* I. The transgenic pTre2-Hand1 construct was isolated from the plasmid backbone by *Aat*II and *Apa*L1 digest. The linearised fragments were purified by phenol/chloroform extraction and ethanol precipitation.

The constructs were made using genomic fragments isolated from entire genomic DNA of the 129Sv mouse strain (Jackson Laboratories, USA). Optimal rates of homologous recombination are achieved when the DNA of the targeting vector and ES cell genome are isogenic. For this reason, the targeting constructs were electroporated into 129Sv-derived ES cells. Two independent electroporations were carried out for *Tet-Off-Hand1*, according to genOway's standard procedures (260 Volt, 500 μF, $5x10^6$ cells for 40 μg of linearised targeting vector). Three separate electroporations were performed for *Tre2-Hand1-EGFP*. Two were carried out as described above, from which only very few resistant clones were obtained. A third was, therefore, undertaken using altered conditions of 800 Volt, 3 μF, $57x10^6$ cells for 67 μg of linearised DNA. One electroporation was carried out for the transgenic construct *Tre2-Hand1*, according to their standard procedures (see above). A second round of electroporation was carried out for the *Tre2-Hand1* transgene with altered conditions of 800 volt, 300 μF, $100x10^6$ cells for 100 μg of transgene DNA. Recombinant clones stably expressing the neomycin resistance cassette, therefore presumably with stable integration of the

appropriate construct, were selected for by adding 200 μg/ml of G418 (synthetic neomycin) after 48 hours, except those produced by the third round of electroporation for *Tre2-Hand1-EGFP*, which were selected at 150 μg/ml, and those produced by the second round for *Tre2-Hand1* that were selected with the addition of 125 μg/ml. Resistant clones were amplified in duplicate in 24-well plates. The duplicate set was used for screening to identify homologous recombination events or transgene integration, while the template set was stored at –80°C for further amplification of any positive clones.

2.2.4 Screening for homologous recombination

In the case of the two targeting constructs, *Tet-Off-Hand1* and *Tre2-Hand1-EGFP*, 502 neomycin resistant ES cell clones in total (239 and 263 respectively) were screened for homologous recombination (heterozygotes) by Southern blotting and PCR.

Tet-Off-Hand1 and *Tre2-Hand1-EGFP* targeted ES cell DNA was digested with *Bgl*II (see Figure 2.1) for 5' probe hybridisations. A 2 kb *Eco*R1-*Kpn*1 probe (see Figure 2.1) was used flanking the 5' end of the constructs.

Further Southern blot analyses were carried out on identified heterozygotes using a *Pst*1 digest and an *Eco*R1-*Eco*R1 2 kb probe (Figure 2.1) to the 3' end of the constructs.

The targeted ES cells were also screened by 3-primer PCR. Primers A, B and C (PrA, PrB and PrC - Figure 2.1) (see Appendix 2) were used for *Tet-Off-Hand1* targeted cells to produce 440 bp wildtype and 690 bp mutant predicted bands. The *Tet-Off-Hand1* construct and *Hand1* wildtype murine DNA were included in the PCR as mutant and wildtype controls respectively. PCR was also performed for *Tre2-Hand1-EGFP* targeted cells using Primers D, E and F (PrD, PrE and PrF - Figure 2.1) (see Appendix 2).

Clones that were identified as correctly targeted were amplified by genOway from the frozen plates and sent for confirmation by a second round of Southern blot and PCR analyses.

2.2.5 Screening for transgene copy number

In the case of the *Tre2-Hand1* transgenic responder, a total of 146 G418 resistant clones (100 first round; 46 second round) were screened for transgene copy number by Southern blot.

DNA was extracted from ES cell clones and Southern blotting was performed. A *Hind*III digest produces a 6.3 kb endogenous *Hand1* band, and a 1.8 kb transgene band (Figure 2.1). The probe is a *Hind*III and *Xho*1 fragment of the transgenic *Tre2-Hand1* construct (Figure 2.1D). Films were scanned and band intensity was quantified by densitometry using the Scion Image program (Scion Corporation) (http://www.scioncorp.com/frames/fr_scion_products.htm). Transgene copy number was assessed relative to the two endogenous copies present in each clone.

Selected clones, and their respective flanking clones, were amplified from frozen stocks and sent to us for confirmation by Southern blot.

2.2.6 Genomic DNA extraction from ES cells

The 24-well plates were thawed and 750 µl of TNE buffer (see Appendix 1) was added to each well. The plates were placed in a 37°C oven for 30 minutes. The cells were thoroughly resuspended in the TNE buffer and transferred to sterile eppendorfs. The cells were then lysed in 0.5% SDS and 0.25 µg/µl proteinase K, at 55°C for three hours. The DNA was extracted in one volume of phenol/chloroform on a nutator, for a minimum of 30 minutes. The extraction mix was centrifuged at 16,000g for 10 minutes at 4°C and the aqueous phase transferred to a fresh eppendorf. If the DNA appeared heavily contaminated the phenol/chloroform extraction step was repeated. The DNA was then precipitated with 0.6 volumes of isopropanol, on a nutator, for a minimum of 30 minutes, until the DNA was visible as a small white pellet. The precipitate was spun down at 16,000g for 10 minutes at 4°C. The pellet was washed in 70% ethanol and resuspended in 50 – 100 µl of TE pH8.0 (see Appendix 1) depending on pellet size and stored at –70°C.

2.2.7 Southern blotting

Targeted ES cell DNA was digested with *Bgl*II, *Pst*1 or *Hind*III for 5' probe, 3' probe and transgene hybridisations, respectively. Approximately 10 µg of DNA were digested

in 1x restriction enzyme buffer containing 1 mM spermidine, 0.1 μg/μl BSA, 75 U enzyme and water up to a final volume of 30 μl. Wildtype (+/+) and *Hand1* heterozygous (+/–) DNA samples were included as controls. The digests were incubated at 37°C overnight. The digests were pulsed down and 6 μl of 6x loading buffer was added prior to running on a 0.6% agarose gel. Samples were run for an hour at 80-100v then at 40-50v overnight.

The gel was visualised under UV light, aligned with a ruler alongside the 1 kb ladder lane and from the top of the wells in order to accurately size resulting bands, and photographed.

The gel was cut into two halves along the well lines, and corners cut for orientation. The gel was acid-linked in acid solution (see Appendix 1) for 10 minutes on a rocking platform at room temperature, to improve the resolution of large fragments in the range 9 kb-13 kb. The gel was then washed in water for five minutes, and denatured with two 30-minute incubations in denaturing solution (see Appendix 1) on a rocking platform.

The DNA was then blotted onto positively charged nylon membrane (Hybond^{TM}N+) using the crush blot method. The two halves of gel were carefully inverted onto a glass plate so that the DNA side was 'up'. Hybond^{TM}N+ membrane and four Whatman sheets per half gel were cut to size and soaked in denaturing solution. The membrane was laid on the gel and rolled to remove any air bubbles. Then the four filter paper sheets were placed on top and rolled again, followed by a three cm stack of paper towels. A glass plate and a weight were put on top of the towels and the blotting apparatus left for five to six hours to ensure complete transfer of DNA onto the membrane. The blot was disassembled and the crushed gel halves were checked under UV light to confirm complete transfer. The membranes were neutralised in neutralising solution (see Appendix 1) for 15 minutes on a rocking platform. The DNA was crosslinked onto the membrane by either baking at 80°C for one hour or UV crosslinking using a Stratagene UV Stratalinker 2400 on the autocrosslink setting (120,000 μjoules/cm^2). The membranes were then stored overnight in a sealed plastic bag at –70°C.

The blots were pre-hybridised at 42°C in 25 ml hybridisation solution (see Appendix 1) for between two and six hours in a bottle rotator hybridisation oven. The probe was radiolabelled by random prime labelling to incorporate α^{32}P-dCTP (see section 2.2.8),

denatured for 10 minutes at 100°C and added directly to the bottle containing the membrane and fresh hybridisation solution. The probe was hybridised at 42°C overnight.

The membranes were washed in the hybridisation bottles (see Appendix 1 for wash solutions). First, the excess probe was thoroughly rinsed out with low stringency wash solution. Then a series of 15-20 minutes washes were carried out as follows:

Two low stringency	at 42°C
Two high stringency	at 42°C
Two high stringency	at 55°C
Two high stringency	at 65°C

A further high stringency wash was carried out at 65°C for 45 minutes to one hour, prior to checking the counts (count per second – cps) on the membrane with a Geiger counter. Optimally between five and ten cps tensured low background. If the counts were high, high stringency washes were carried out at 70°C until the background was sufficiently reduced. The membrane was wrapped in Saran Wrap to prevent drying out, and exposed to film at –70°C. Exposure times varied but typically films were developed after 72 hours.

2.2.8 Random Prime Labelling

Probes for Southern blotting were obtained from digests of 5' and 3' *Handl* genomic clones (sites shown in Figure 2.1A) for screening for homologous recombination, or from a digest of the transgenic responder construct (Figure 2.1D) to screen for transgene copy number. Digested probes were purified using the band purification method. Approximately 40 ng DNA was mixed with 500 ng of random primers (Promega) in water to a total volume of 20 µl. This mixture was heat denatured at 100°C for five minutes and snapped cool on ice for two to three minutes. The labelling reaction was carried out in a volume of 50 µl consisting of 1x random prime reaction buffer (RPRB) (see Appendix 1), 0.4 µg/µl BSA, 10 U Klenow and 1.85 MBq α^{32}P-dCTP. The labelling mix was incubated at 37°C for two hours. The reaction was stopped with 300 µl of 0.1xTE pH8.0/0.1% SDS and the labelling mix spun down a Sephadex G50/TE pH8.0 column to isolate the labelled probe and remove unincorporated ^{32}P.

2.2.9 PCR genotyping

PCR was carried out using puRe Taq™ Ready-To-Go™ PCR beads (Amersham Biosciences), with 10 pmol of each primer and 1 µl of extracted DNA. The PCR reaction consisted of a denaturation step of two minutes at 94°C, then 30 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 45 seconds. The PCR reaction was performed using a PE Applied Biosystems GeneAmp® PCR System 9700.

Primers A, B and C (Figure 2.1) were used for *Tet-Off-Hand1* genotyping. Primers D, E and F (Figure 2.1) were used for *Tre2-Hand1-EGFP* genotyping. Primers G and H were used for *Tre2-Hand1* genotyping (see Appendix 2 for primer sequences).

2.2.10 Recombinant ES cell injections into blastocysts (genOway)

Two correctly targeted clones identified for each targeting construct and 14 clones identified for the transgenic construct were subsequently used for blastocyst injection with the aim of generating chimeric mice. Recipient blastocysts were isolated from pregnant wildtype C57BL/6 SPF (specific pathogen free) females. The recombinant clones were injected into the day 3.5 blastocysts and cultured overnight. The injected blastocysts were then implanted into pseudo-pregnant females (health status SOPF (specific and opportunistic pathogen free)). For *Tet-Off-Hand1*, clone 10 was injected into 52 blastocysts that were implanted into four pseudo-pregnant females, clone 84 was injected into 75 blastocysts and implanted in five pseudo-pregnant females (see Table 2.1). For *Tre2-Hand1-EGFP*, clone 48 was injected into 52 blastocysts, implanted into three pseudo-pregnant females and clone 159 was injected into 51 blastocysts, implanted into three pseudo-pregnant females (see Table 2.2).

Table 2.1. ***Tet-Off-Hand1 injected blastocysts and implanted females***

Clone number	Injected blastocysts	Implanted females
10	52	4
84	75	5
Total	**127**	**9**

Table 2.2. ***Tre2-Hand1-EGFP injected blastocysts and implanted females***

Clone number	Injected blastocysts	Implanted females
48	51	3
159	52	3
Total	**103**	**6**

For *Tre2-Hand1*, clone 32 was injected into 28 blastocysts, clone 36 was injected into 26 blastocysts, clone 39 was injected into 25 blastocysts, clone 99 was injected into 27 blastocysts and clone 100 was injected into 31 blastocysts. Blastocysts were implanted into two pseudo-pregnant females per individual clone (see Table 2.3). Due to only two transgenic lines being established through the first set of blastocyst injections, and the lack of a line with a copy number of two, a further 75 injected blastocysts were implanted into five pseudo-pregnant females for clone 100 (see Table 2.3). Additional clones with a copy number of two were also thawed and injected into blastocysts, a total of 533 blastocysts were injected into 37 pseudo-pregnant females (see Table 2.3). Following the failure of any of the previously identified copy number two clones to contribute significantly to any chimeras born, a second round of electroportation was undertaken identifying new clones to be used for blastocyst injection. Clone 26 was injected into 45 blastocysts and implanted into three pseudo-pregnant females, clone 32 and clone 46 were each injected into 30 blastocysts and implanted into two females (see Table 2.4).

Table 2.3. ***Tre2-Hand1 injected blastocysts and implanted females***

Clone number	**Blastocysts injected**	**Implanted females**
13	15	1
23	49	4
32	28	2
36	26	2
37	88	6
38	147	10
39	25	2
45	30	2
58	30	2
80	75	5
83	69	5
91	30	2
99	27	2
100	106	7
Total	**745**	**52**

Table 2.4. ***Tre2-Hand1 injected blastocysts and implanted females (second round)***

Clone number	**Injected blastocysts**	**Implanted females**
26	45	3
32	30	2
46	30	2
Total	**105**	**7**

2.2.11 Chimera breeding: Generation of heterozygous mice (genOway)

Various percentage chimeras were produced for all constructs. Chimerism was assessed on the basis of coat colour. The ES cells are derived from the 129 mouse strain and transmit agouti coat colour. Targeted ES cells were injected into blastocysts harvested from wildtype C57Bl/6 females (black coat colour). Percentage chimerism in resultant offspring, therefore, was determined by extent of ES cell contribution assessed in turn by coat colour. The more agouti the mice, the higher the contribution from the ES cells and consequently the higher the percentage chimerism.

The four highest percentage male *Tet-Off-Hand1* chimeras were put into breeding for a seven week period with wildtype C57BL/6 females to check for germline transmission to the F1. The rationale is that it is hoped that the targeted ES cells in the highest percentage males should contribute significantly to the testis and gamete production. All male *Tre2-Hand1-EGFP* chimeras plus three female chimeras were put into breeding with wildtype C57BL/6 mice. For the first set of *Tre2-Hand1* lines, breeding with wildtype C57BL/6 mice was carried out with the 100% chimera from clone 39, the three 100% chimeras from clone 36, the 40% chimera from clone 32, the 30% chimera from clone 83 and the 5% chimera from clone 100. For the second set of *Tre2-Hand1* lines, the 20% chimera from clone 26, the 65% chimera from clone 32 and the 65% and 90% chimeras from clone 46 were put into breeding with C57BL/6 wildtypes. Tail biopsies of any offspring were sent to us for characterization of heterozygous mice by southern blot and PCR. Identified heterozygotes and original chimeras were shipped to the in-house animal facility to begin expanding the colony.

2.2.12 Genomic DNA extraction from mouse tail tip

2.2.12.1 For Southern blotting to identify heterozygotes

DNA was extracted from mouse tail tips in the same way as from ES cells (see section 2.2.6), in 1 ml TNE, an overnight incubation at 55°C and with an extra chloroform extraction step. The DNA was resuspended in 100 µl TE.

2.2.12.2 For PCR genotyping for maintenance of colony

Tail tips were lysed in 500 µl lysis buffer (see Appendix 1) at 55°C overnight. DNA was precipitated with an equal volume of isopropanol, and washed in 70% ethanol. The pellet was resuspended in 200-400 µl depending on pellet size.

2.3 Results

2.3.1 *Tet-Off-Hand1*

2.3.1.1 Identification of homologous recombinants

genOway provided 239 G418 resistant ES cell clones (89 from the first electroporation event, 150 from the second) frozen in 24-well plates. DNA was extracted and Southern blotting performed, initially using a 5' flanking probe (Figure 2.1A). Heterozygote clones (homologous recombinants) were predicted to have a wildtype band of 13 kb, and a mutant band of 9 kb (Figure 2.3A). 116 ES cell clones were screened identifying 17 heterozygotes (clones 3, 10, 17, 22, 34, 40, 42, 60, 62, 69, 75, 81, 84, 93, 95, 105 and 116). These were confirmed by further Southern blotting using a 3' probe (data not shown) and by PCR (Figure 2.3B). Seven clones (3, 10, 17, 40, 42, 84, 95) along with their respective flanking clones were amplified from frozen stocks at genOway and sent to us for confirmation by Southern blot. Clones 10 and 84 (Figure 2.3A) were selected, as correctly targeted by homologous recombination for the *Tet-Off-Hand1* construct, to be used for blastocyst injection and generation of chimeric mice.

2.3.1.2 Blastocyst injection

Clones 10 and 84 were injected into blastocysts and implanted into pseudo-pregnant females. All the nine implanted females became pregnant and had a total of 71 pups, 36 of which were from clone 10, 35 from clone 84. Of the 71 pups, there were 11 male chimeras (nine from clone 10, two from clone 84) and 5 female chimeras (two from clone 10, three from clone 84). The chimeras ranged from 3% to 98% ES cell contribution (Figure 2.4), the results are summarised in Table 2.5.

2.3.1.3 Chimera breeding to generate F1 heterozygous mice

Four chimeric males were chosen based on their high degree of chimerism to be put into breeding with wildtype C57BL/6 females in order to generate heterozygote F1 and confirm transmission of the *Tet-Off-Hand1* construct through the germline. The males used were 98%, 95% and 95% derived from clone 10 and 90% derived from clone 84. Germline transmission was assessed initially using pup coat colour. Agouti colour pups are derived from the targeted ES cells with an expected 1:1 ratio of wildtype to heterozygote for the *Tet-Off-Hand1* allele (*Hand1*^{tTA}) in any agouti litters.

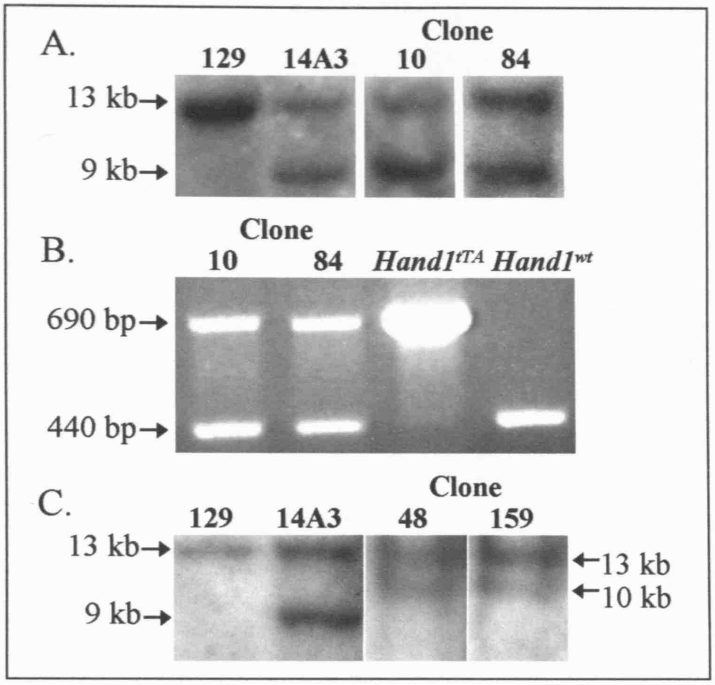


Figure 2.3. Southern blot and PCR analyses to determine homologous recombinants. The constructs were sent to genOway, a commercial company, for targeting. 129Sv ES cells were electroporated and selected for neomycin resistance. Resistant clones were screened for homologous recombination (heterozygotes) by southern blotting and PCR. *Tet-Off-Hand1* (A) and *Tre2-Hand1-EGFP* (C) targeted ES cell DNA was digested with *Bgl*II (Figure 2.1) for 5' probe hybridisations. A 2 kb *Eco*R1-*Kpn*1 probe (Figure 2.1) was used flanking the 5' end of the constructs. Clone 10 and clone 84 were identified as correct homologous recombinants for the *Tet-Off-Hand1* construct having a predicted wild type band of 13 kb, and a mutant band of 9 kb (A). Clone 48 and clone 159 were identified for *Tre2-Hand1-EGFP* construct, with the same 13 kb wildtype band and a mutant band of 10 kb (C). 129 (wildtype) and 14A3 (heterozygous) DNA samples were included as controls. The targeted ES cells were also screened by three-primer PCR. PrA, PrB and PrC (Figure 2.1) were used for *Tet-Off-Hand1* targeted cells to produce 440 bp wildtype and 690 bp mutant predicted bands. Clone 10 and clone 84 were confirmed as homologous recombinants alongside the *Tet-Off-Hand1* construct and wildtype genomic DNA as controls (B). PCR was also performed for *Tre2-Hand1-EGFP* targeted cells using PrD, PrE and PrF (see Figure 2.1; data not shown).

See Figure 2.3.1 for full restriction map

Figure 2.3.1. Detailed map showing restriction sites used for Southern blotting. (A) The endogenous *Hand1* locus, (B) the *Tet-Off-Hand1* targeting construct, (C) the *Tre2-Hand1-EGFP* targeting construct and (D) the *Tre2-Hand1* transgenic construct. The restriction sites used and the corresponding expected fragment sizes are shown in red. The three constructs were electroporated into ES cells and selected clones were screened by Southern blot (see sections 2.3.1.1, 2.3.2.1 and 2.3.3.1). ES cell DNA from selected clones for the two targeting constructs (B and C) was digested with *Bgl*II for 5' probe hybridisations. This generates expected fragment sizes of 13 kb from the endogenous *Hand1* locus (A), and 9 kb and 10 kb from the correctly targeted *Tet-Off-Hand1* (B) and *Tre2-Hand1-EGFP* (C) constructs respectively. ES cell DNA from selected clones for the transgenic *Tre2-Hand1* construct (D) was digested with *Hind*III for hybridisations using the probe indicated in D. This generates expected fragment sizes of 6.3 kb from the endogenous *Hand1* locus (A) and 1.6 kb from the *Tre2-Hand1* transgenic construct (D).

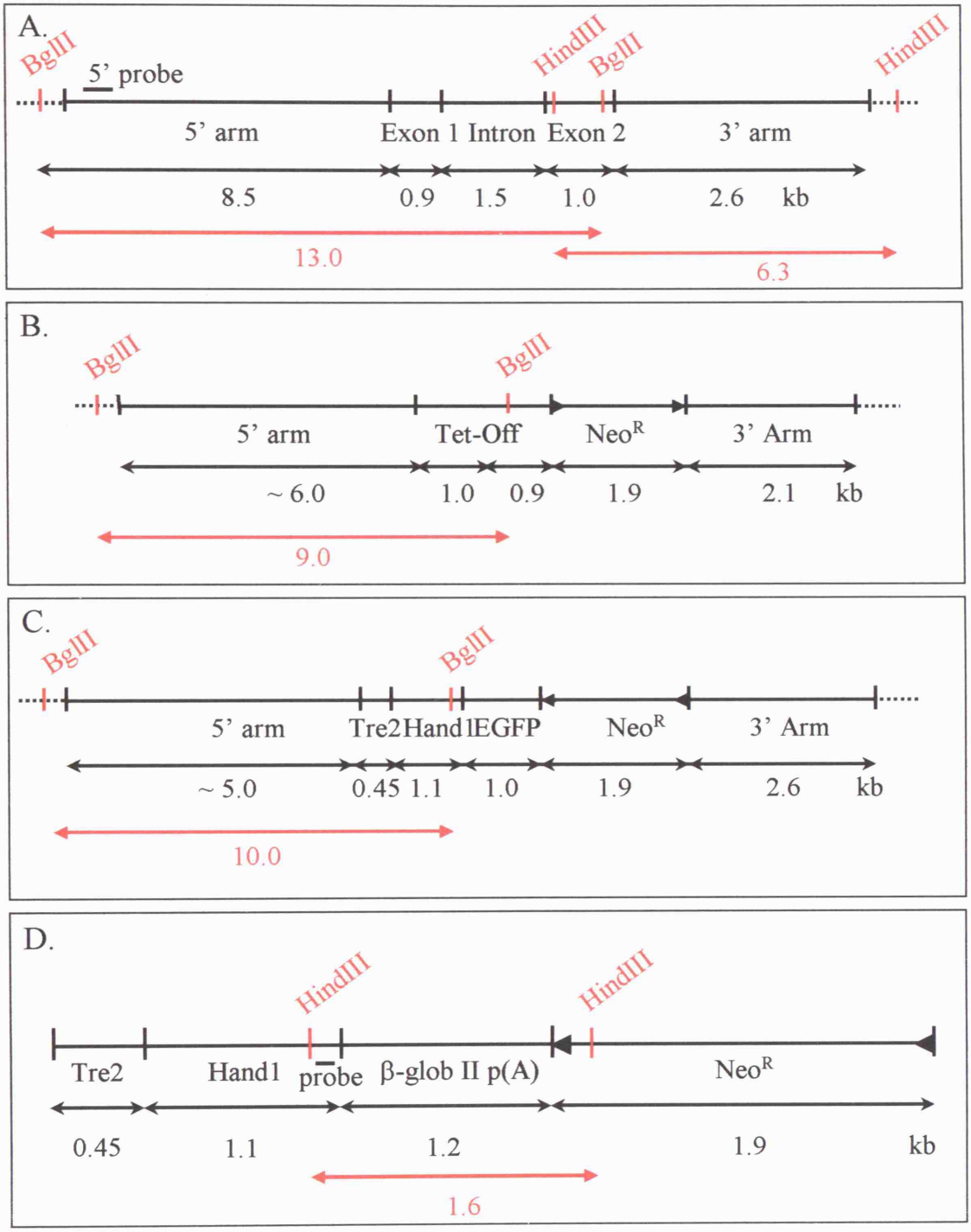

A.
BglII
5' probe
HindIII
BglII
HindIII
5' arm
Exon 1
Intron
Exon 2
3' arm
8.5
0.9
1.5
1.0
2.6
kb
13.0
6.3
B.
BglII
BglII
5' arm
Tet-Off
NeoR
3' Arm
~ 6.0
1.0
0.9
1.9
2.1
kb
9.0
C.
BglII
BglII
5' arm
Tre2
Hand1
EGFP
NeoR
3' Arm
~ 5.0
0.45
1.1
1.0
1.9
2.6
kb
10.0
D.
HindIII
HindIII
Tre2
Hand1
probe
β-glob II p(A)
NeoR
0.45
1.1
1.2
1.9
kb
1.6

Table 2.5. ***Tet-Off-Hand1 results of blastocyst injection to generate chimeras***

Clone number	Injected blastocysts	Implanted (pregnant) females	Total pups	Male chimeras (%)	Female chimeras (%)
10	52	4 (4)	36	9 (3, 20, 40, 50, 70, 95, **95**, **95**, and **98**)	2 (15 and 40)
84	75	5 (5)	35	2 (**90** and 75)	3 (85, 80 and 10)
Total	**127**	**9 (9)**	**71**	**11**	**5**

Chimeras shown in bold were subsequently used for breeding with wildtype C57BL/6 to check for germline transmission.

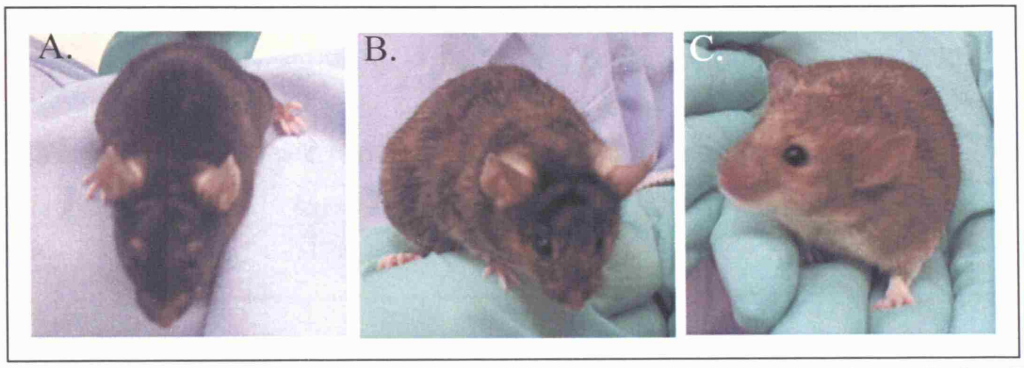


Figure 2.4. Chimeric mice derived from a parent C57Bl/6 strain and the *Tet-Off Hand1* targeted ES cell lines showing varying degrees of targeted ES cell line contribution. (A) 3%, (B) 50% and (C) 98%. The ES cells are derived from the 129 mouse strain and transmit agouti coat colour. Targeted ES cells were injected into day 3.5 blastocysts harvested from wildtype C57Bl/6 females (black coat colour). The blastocysts were cultured overnight and transferred into C57Bl/6 pseudo-pregnant females. Percentage chimerism in resultant offspring was determined by extent of ES cell contribution assessed in turn by coat colour. The more agouti the mice, the higher the percentage chimerism. The highest percentage male chimeras (95%-98%) were put into breeding with wildtype C57Bl/6 females to produce the F1 generation and check for germline transmission of the *Tet-Off Hand1* construct. Germline transmission has been obtained with the 98% male (C) derived from targeted clone 10 (see Figure 2.3).

Two male chimeras (95% clone 10 and 90% clone 84) appeared to be sterile as they produced no offspring during the seven week breeding period. The ES cells used for the generation of the mouse strains described here, and also the majority of existing ES cell lines, have the genotype XY. If the ES cells are injected into a blastocyst of the genotype XX, the resultant chimera will comprise a mixture of XX and XY cells.

Determination of percentage chimerism and gender are based upon external cues of anatomy and coat colour respectively. However, the internal reproductive system of a chimeric mouse could be composed of the opposite genotype, or a mixture of female and male genotype. Chimera sterility is, therefore, to be expected in some cases.

A second 95% clone 10 derived chimera produced four black litters; one of these litters consisted of four out of six stillborn offspring. The fourth clone 10 chimera (98%) produced five agouti litters. The first 11 agouti pups born were tail tipped and the lysates sent to us to screen for heterozygotes. The DNA was extracted and Southern blot (5' and 3' probe), and PCR was performed. Five out of the 11 were identified as heterozygotes, one female and four males, which conforms to the expected 1:1 ratio. The complete breeding results are listed in Table 2.6.

Table 2.6. ***Tet-Off-Hand1 results of chimera breeding to produce the F1 generation***

Male chimera	Number of pups	Coat Colour	Sex ratio
98% clone 10	36	Agouti	17 males 15 females
95% clone 10	24	4 stillborn 20 Black	-

Germline transmission has been obtained with the 98% male (Figure 2.4C) derived from targeted clone 10 (Figure 2.3A).

The five identified heterozygotes and original chimeras were shipped to us to begin expanding the colony.

In an attempt to obtain a second line for the *Tet-Off-Hand1* strain, the two clone 84 chimeras (90% and 75%) were put into breeding with C57BL/6 females in-house before making a final decision on successful or failed germline transmission. The 90% male chimera appeared to be sterile as he continued to fail to produce any litters. The 75% male chimera, previously untested, produced four black, wildtype litters.

Three F1 males derived from the transmitting 98% clone 10 chimera were backcrossed with C57BL/6 females, and the remaining male was crossed with the female F1 heterozygote to produce the F2. In addition, the 98% male chimera was bred with wildtype C57BL/6 to produce more F1 to help expand the colony.

2.3.2 *Tre2-Hand1-EGFP*

2.3.2.1 Identification of homologous recombinants

genOway provided 263 G418 resistant ES cell clones frozen in 24-well plates. DNA was extracted and Southern blotting performed, initially using a 5' flanking probe (Figure 2.1A), as for *Tet-Off-Hand1*. Heterozygotes (homologous recombinants) were predicted to have a wildtype band of 13 kb, and a mutant band of 10 kb (Figure 2.3C). All 263 ES cell clones were screened identifying three possible heterozygotes (clones 44, 48, 159). Further southern blotting using a 3' probe (Figure 2.1A) was carried out to attempt to confirm homologous recombination, along with PCR (not shown). The 3' Southern blot and PCR confirmed only clone 48. Clone 159 gave no result suggesting a possible rearrangement at the 3' end of the construct during recombination. Clone 44 was confirmed as incorrectly targeted. All three clones were sent to us for validation along with their respective flanking clones, producing the same results. To maximise the chance of germline transmission, clone 159 was included with clone 48 for blastocyst injection and subsequent generation of chimeric mice, since it was assumed the 5' arm plus the *Tre2-Hand1-EGFP-neo*^{r} component of the construct had recombined at the *Hand1* locus.

2.3.2.2 Blastocyst injection

Both clones 48 and 159 were injected into blastocysts and implanted into pseudo-pregnant females. Three pseudo-pregnant females were implanted for each clone. All three implanted females for clone 159 became pregnant and had a total of 25 pups. Five of these were chimeric, three males and two females, but all at low levels of contribution (5-15%) and the 15% male was abnormally small and died two weeks after birth. However, only one of the three re-implanted females for clone 48 became pregnant, and had a litter of nine pups of which four were chimeric, one male and three females, all at 70% chimerism. The results for the *Tre2-Hand1-EGFP* construct chimera generation are summarised in Table 2.7.

Table 2.7. ***Tre2-Hand1-EGFP results of blastocyst injection to generate chimeras***

Clone number	Injected blastocysts	Implanted (pregnant) females	Total pups	Male chimeras (%)	Female chimeras (%)
48	52	3 (3)	25	3 (**5**, **10**, 15*)	2 (10 and 15)
159	51	3 (1)	9	1 (**70**)	3 (**70**)
Total	**103**	**6 (4)**	**34**	**4**	**5**

Chimeras shown in bold were subsequently used for breeding with wildtype C57BL/6 to check for germline transmission. *15% male chimera from clone 159 died two weeks after birth and was abnormally small.

2.3.2.3 Chimera breeding to generate F1 heterozygous mice

All four male chimeras and the 70% female chimeras were put into breeding with wildtype C57BL/6 mates to check for germline transmission and generate heterozygotes. Germline transmission, as before, was estimated based on transmission of agouti coat colour. All the chimeras had between two and five litters in genOway care and all pups were black, some litters were stillborn. The six chimeras used for breeding F1 were shipped to the Western Labs (in house) to continue breeding the 70% chimeras. The male and each female had a further three litters each but all pups were black. Germline transmission was not obtained for the *Tre2-Hand1-EGFP* construct.

2.3.2.4 Second round of ES cell targeting

As germline transmission was not achieved for the *Tre2-Hand1-EGFP* targeting vector, a second round of blastocyst injection was scheduled to be performed, using ES cells grown and amplified from the frozen stocks of clones 48 and 159. Upon thawing, genOway observed that the cell morphology and growth rate were totally abnormal and decided that an entirely new round of ES cell electroporation would be required.

ES cells were electroporated and selected as before and 239 G418 resistant clones were sent to us frozen on 24-well plates to screen for homologous recombination. DNA was extracted and Southern blotting hybridising with the 5' probe was performed in the same way as described under section 2.3.2.1. 164 ES cell clones were screened in total but no correctly targeted clones were identified. At this point, the targeting of the *Tre2-Hand1-EGFP* responder to the endogenous *Hand1* locus was abandoned in favour of random integration of a transgenic version, *Tre2-Hand1*.

2.3.3 *Tre2-Hand1*

2.3.3.1 Identification of positive clones and transgene copy number

Following standard ES cell electroporation, genOway provided 119 G418 resistant ES cell clones frozen on 24-well plates and DNA was extracted. A total of 100 clones were screened by Southern blotting using a *Hand1* probe (see Figure 2.1D) and copy number was assessed. 41 clones were identified that carried between one and 79 copies of the transgene (see Table 2.8). Five clones (47, 32, 99, 39, 36 having a copy number of 2, 3, 4, 5 and 12 respectively, see Table 2.8 and Figure 2.5) were selected. These five clones, and their flanking clones, were amplified from frozen stocks at genOway and sent for confirmation by Southern blot.

Clones 32, 99, 39 and 36 were confirmed as having the correct transgene copy number, whilst clone 47 appeared to have an elevated copy number relative to the first Southern blot screen. Clone 100 was chosen to replace clone 47 for copy number two. The five confirmed clones for the transgenic *Tre2-Hand1* construct were used for blastocyst injection and generation of chimeric mice.

Table 2.8. ***Positive Tre2-Hand1 ES cell clones and respective transgene copy number***

Clone	Copy number	Clone	Copy number	Clone	Copy number	Clone	Copy number
2	12	38	2	54	1	86	1
7	66	**39**	**5**	56	18	87	1
13	2	45	2	58	5	88	35
18	6	46	2	59	1	91	7
23	2	47	2	60	1	93	79
30	17	48	2	78	19	95	16
31	1	49	2	79	22	96	14
32	**3**	51	1	80	2	98	39
34	13	52	1	81	36	**99**	**4**
36	**12**	53	1	83	2	**100**	**2**
37	2						

The ES cell clones highlighted in bold were chosen initially for injection into blastocysts and generation of chimeric mice. The ES cell clones in blue were chosen subsequently for attempts to generate the third transgenic line.

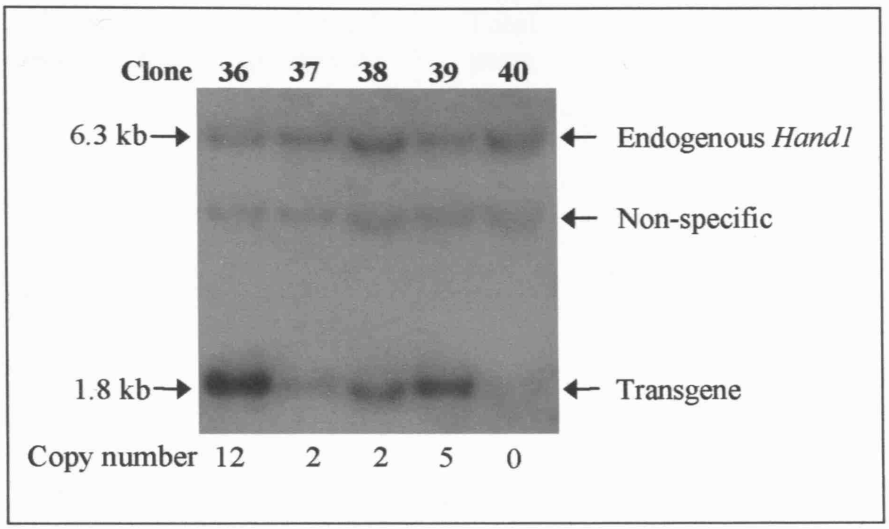


Figure 2.5. Southern blot analyses to determine transgene copy number. Neomycin resistant transgenic 129Sv ES cell clones were generated as in Figure 2.3. Resistant clones were screened for transgene integration by Southern blotting. *Tre2-Hand1* ES cell DNA was digested with *Hind*III (see Figure 2.1). A 0.5 kb *Hind*III-*Xho*1 probe (see Figure 2.1) encompassing exon 2 and the 3'UTR of *Hand1* was used. Clones 36 to 40 are shown, having a predicted wildtype endogenous band of 6.3 kb, and a transgene band of 1.8 kb. Note the varying copy numbers. Clone 36 (copy number 12) and clone 39 (copy number five) were two of the ES cell clones selected for generation of transgenic strains, along with three other clones (not shown) having copy numbers of two, three and four.

2.3.3.2 Blastocyst injection

The five clones were injected into blastocysts and implanted into pseudo-pregnant females. Eight of the ten implanted females became pregnant and had a total of 31 pups. Of these 31 pups, there were eight male chimeras from four of the five clones (one from clone 32, five from clone 36, one from clone 39, one from clone 100) and two female chimeras (both from clone 36). Clone 99 failed to produce any chimeric pups. The chimeras ranged from 5% to 100% ES cell contribution, and the results are summarised in Table 2.9.

Table 2.9. ***Transgenic Tre2-Hand1 results of blastocyst injection to generate chimeras***

Clone number	Injected blastocysts	Implanted (pregnant) females	Total pups	Male chimeras (%)	Female chimeras (%)
32 (3)	28	2 (2)	9	1 **40%**	0
36 (12)	26	2 (2)	11	5 10%, 20% **3 x 100%**	2 95%, 100%
39 (5)	25	2 (1)	1	1 **100%**	0
99 (4)	27	2 (2)	6	0	0
100 (2)	31	2 (1)	4	1 **5%**	0
Total	**137**	**10 (8)**	**31**	**8**	**2**

The chimeras highlighted in bold were used for subsequent breeding with wildtype C57BL/6 to check for germline transmission of transgene. Transgene copy number is shown in brackets next to the clone number.

2.3.3.3 Chimera breeding to generate F1 mice carrying the transgene

In the first instance, all of the chimeras (except the 10% and 20% from clone 36) were put into breeding with wildtype C57Bl/6 females to check for germline transmission of the transgene. As before, germline transmission was first assessed based upon transmission of agouti coat colour.

The clone 100 5% chimera appeared to be sterile, as did one of the clone 36 100% chimeras, producing no litters. The clone 39 100% chimera had one litter of all black offspring. The clone 32 40% chimera had three litters in genOway care, the first of which provided two black pups, but the subsequent two litters were each of two agouti pups. The remaining two 'clone 36' 100% chimeras both produced litters of agouti offspring. All the agouti offspring were tail tipped and the lysates shipped to us for confirmation of transgene transmission. DNA was extracted and screened by PCR and Southern blot. For clone 36, 17 out of 34 were identified as carrying the correct 12 copies of the transgene, whilst for clone 32 one out of four was identified as having three copies, each conforming to the expected 1:1 ratio of wildtype to transgenic progeny. The complete breeding results are listed in Table 2.10.

Germline transmission has been obtained for clone 32 (copy number 3; *Tre2-Hand1*(CN3)) and clone 36 (copy number 12; *Tre2-Hand1*(CN12)).

The 17 transgenic pups and the parent chimeras were then shipped to us. The three (two clone 36, one clone 32) chimeras and also two clone 36 F1 males and the single clone 32 F1 female were immediately crossed into the *Hand1* heterozygote background using the *Hand1* targeted mice available in house.

Table 2.10. ***Tre2-Hand1 results of chimera breeding to produce the F1 generation***

Male chimera	Number of pups	Coat Colour	Sex ratio
40% clone 32	2	Black	-
	4	Agouti	
100% Clone 36		Agouti	
100% Clone 36		Agouti	
100% Clone 36	None	-	-
100% Clone 39	5	Black	-
5% Clone 100	None	-	-

2.3.4 Generation of a third *Tre2-Hand1* line

One reason for choosing ES cells to generate transgenic mice was that copy number of transgene could be selected for, and several lines with potentially varying levels of expression of transgene could be established (see section 2.1.2). These lines could then be tested for appropriate levels of expression comparable to that of endogenous *Hand1*, so that *Hand1* expression is normal in the compound heterozygotes, and not over-expressed. Therefore, although two copies of the *Tre2-Hand1* transgene may not translate to the same level of *Hand1* expression as two wildtype copies, it seemed a reasonable starting point to generate a line with two copies of *Tre2-Hand1*. After repeatedly failing to generate chimeras with a significant ES cell contribution with several copy number two ES cell clones, the process was also attempted using two ES cell clones that had a higher copy number. It is preferable to examine multiple transgenic lines to exclude potential artefacts caused by integration site, therefore, generation of a third line, with any copy number, was important.

2.3.4.1 Blastocyst injection

Following the failure of the 5% chimera from clone 100 to produce any litters, blastocyst injection was repeated for clone 100. In addition, numerous additional blastocyst injections were performed and implanted into pseudo-pregnant females in an

attempt to generate a third transgenic line. Initially 'new' clones were selected based upon having a transgene copy number of two. A further two clones; clone 58 (copy number five) and clone 91 (copy number seven) were also selected to increase the chances of generating a third line. In total, nine clones (13, 23, 37, 38, 45, 58, 80, 83 and 91; see Table 2.8) with varying copy number were all selected to be used in an attempt to generate a third transgenic *Tre2-Hand1* mouse line. A total of 608 blastocysts were injected into 42 pseudo-pregnant females. 22 of the 42 implanted females became pregnant and had a total of 93 pups. Of these pups, there were 7 male chimeras and 15 were female chimeras. Clones 13, 45, 58 and 91 failed to produce any chimeric pups. The chimeras ranged from 2% to 30% ES cell contribution, and the results are summarised in Table 2.11.

Table 2.11. ***Transgenic Tre2-Hand1 results of subsequent blastocyst injections to generate chimeras for a third transgenic line***

Clone number	Injected blastocysts	Implanted (pregnant) females	Total pups	Male chimeras (%)	Female chimeras (%)
13 (2)	15	1 (0)	-	-	-
23 (2)	49	4 (3)	8	-	1 5%
37 (2)	88	6 (3)	19	1 2%	5 5%, 4 x 2%
38 (2)	123	10 (3)	13	2 5%, 2%	2 2 x 10%
45 (2)	30	2 (1)	4	-	-
58 (5)	30	2 (0)	-	-	-
80 (2)	75	5 (4)	13	5%	-
83(2)	69	5 (4)	17	**30%**	-
91 (7)	30	2 (2)	-	-	-
100 (2)	75	5 (5)	19	2 5%, 2%	7 2 x 5%, 5 x 2%
Total	**608**	**42 (22)**	**93**	**7**	**15**

The chimeras highlighted in bold were used for subsequent breeding with wildtype C57BL/6 to check for germline transmission of transgene. Transgene copy number is shown in brackets next to the clone number.

2.3.4.2 Chimera breeding

Since the ES cell contribution to chimeras was so poor for the majority of clones for which it was attempted, the only chimera put into breeding with a C57/Bl6 wildtype female was the 30% chimera from clone 83. However, this chimera failed to produce any offspring/agouti pups. Following this lack of success using previously identified

ES clones, it was decided to repeat the electroporation with the intention of generating an entirely new set of transgenic *Tre2-Hand1* ES cell clones.

2.3.4.3 Identification of positive clones and transgene copy number

ES cells were electroporated and selected as before and 105 G418 resistant clones were sent to us frozen on 24-well plates to screen for presence of transgene. DNA was extracted and Southern blotting hybridising with the transgene probe was performed in the same way as described under section 2.3.3.1. 17 out the 46 ES clones screened were identified as positives, carrying between one and 11 copies of the transgene. Six clones (20, 26, 32, 34, 42 and 46 having a copy number of 2, 2, 1, 3, 11 and 2 respectively, see Figure 2.6 and Table 2.12) were selected. These six clones, and their flanking clones, were amplified from frozen stocks at genOway and sent for confirmation by Southern blot. All clones were confirmed as having the correct transgene copy number. Three confirmed clones for the transgenic *Tre2-Hand1* construct (clone 26, 32 and 46) were used for blastocyst injection and generation of chimeric mice.

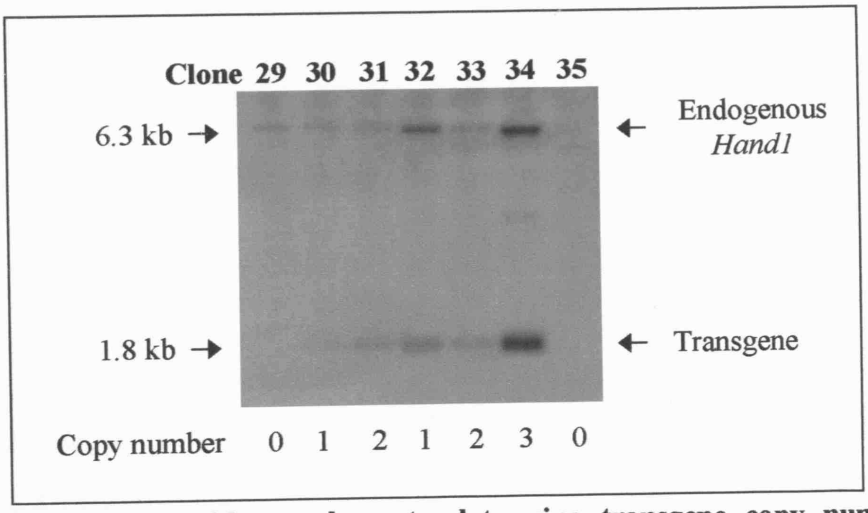


Figure 2.6. Southern blot analyses to determine transgene copy number for generation of the third transgenic *Tre2-Hand1* line. Following several rounds of failed blastocyst injection, the transgenic construct was electroporated into ES cells and selected for neomycin resistance to generate an entirely new set of ES cell clones. Resistant clones were screened for transgene integration by Southern blot as before (see Figure 2.5; DNA was digested with *Hind*III). Clone 32 (copy number one) was selected for generation of transgenic strains, along with two other clones (not shown) both having a copy number of two.

Table 2.12. ***Tre2-Hand1 positive clones and transgene copy number***

Clone number	Copy number	Clone number	Copy number
15	1	31	2
17	1	**32**	**1**
20	2	33	2
21	1	34	3
22	2	39	1
25	2	40	11
26	**2**	42	1
27	2	**46**	**2**
30	1		

The ES cell clones highlighted in bold were chosen for injection into blastocysts and generation of chimeric mice.

2.3.4.4 Blastocyst injection

The three clones were injected into blastocysts and implanted into pseudo-pregnant females. Six of the seven implanted females became pregnant and had a total of 25 pups. Of these pups, there were five male chimeras (one clone 26, one clone 32, three clone 46) and two female chimeras (both from clone 26). The chimeras ranged from 20% to 90% ES cell contribution, and the results are summarised in Table 2.13.

Table 2.13. ***Transgenic Tre2-Hand1 results of blastocyst injection to generate chimeras***

Clone number	Injected blastocysts	Implanted (pregnant) females	Total pups	Male chimeras (%)	Female chimeras (%)
26(2)	45	3 (3)	13	1 **20%**	2 85%, 40%
32(1)	30	2 (2)	5	1 **65%**	-
46(2)	30	2 (1)	7	3 **90%, 65%,** 30%	-
Total	**105**	**7 (6)**	**25**	**5**	**2**

The chimeras highlighted in bold were used for subsequent breeding with wildtype C57BL/6 to check for germline transmission of transgene. Transgene copy number is shown in brackets next to the clone number.

2.3.4.5 Chimera breeding to generate F1 mice carrying the transgene

Four male chimeras were put into breeding with wildtype C57Bl/6 females to check for germline transmission of the transgene. The males used were 20% from clone 26, 65% from clone 32 and 90% and 65% derived from clone 46. As before, germline transmission was based first upon transmission of agouti coat colour.

All chimeras produced offspring, however the clone 32 chimera produced black litters. The clone 26 chimera produced black litters initially, but then produced two agouti females, for which tail biopsies were received to screen for transgene transmission. The two clone 46 chimeras both produced agouti litters, the first 12 agouti pups born were tail tipped and the lysates received to screen for heterozygotes. The DNA was extracted and PCR was performed to confirm the presence of the transgene. Both agouti pups from clone 26, and five out of the 12 clone 46 agouti pups were identified as heterozygotes, which conforms to the expected 1:1 ratio. The complete breeding results are listed in Table 2.14. Again, the transgenic mice were crossed into the *Hand1* heterozygote background for future generation of compound heterozygotes.

Table 2.14. ***Tre2-Hand1 results of chimera breeding to produce the F1 generation***

Male Chimera	Number of pups	Coat colour	Sex ratio
20% clone 26	18 2	Black Agouti	- 2 females
65% clone 32	13	Black	-
90% clone 46	3	Agouti	1 male 2 females
65% clone 46	9	Agouti	6 males 3 females

2.4 Discussion

2.4.1 *Tet-Off-Hand1*

Germline transmission has been successfully obtained for the knocking-in of the *Tet-Off-Hand1* construct into the *Hand1* locus. ES cell electroporation was successful with a targeting efficiency of ~15% and 16 chimeras were generated. The 98% chimera derived from clone 10 transmitted the targeted allele through the germline producing heterozygous mice that were bred to expand the colony in preparation for future use in crosses with the responder line. This is an important mouse line to have generated since it allows for the correct spatial and temporal regulation of the Tet-Off transactivator in accordance with endogenous *Hand1* expression.

2.4.2 *Tre2-Hand1-EGFP*

Germline transmission was not obtained for the *Tre2-Hand1-EGFP* construct. Targeted ES cells were identified, but at a very low targeting efficiency of 0.01%, and nine

chimeras were produced, however all nine failed to pass on the targeted allele to the F1 generation. Unfortunately, a second round of ES cell targeting also failed to identify any homologous recombinants. This, along with reasons discussed in sections 2.1.2 and 2.1.4, prompted the adoption of a transgenic approach for the responder line, as an alternative strategy to further targeting of the endogenous *Hand1* locus.

2.4.3 *Tre2-Hand1*

Germline transmission was obtained for two independent transgenic *Tre2-Hand1* lines. A high number of ES clones were identified as containing the transgene (41/100) and many chimeras were generated. One 100% chimera derived from clone 36 and the 40% chimera derived from clone 32 transmitted the transgene through the germline, producing transgenic mice that were received and used to expand the colony.

Germline transmission was not successfully obtained for a third transgenic line using the initial set of ES cell clones. Repeated attempts at blastocyst injection and implantation into pseudo-pregnant females resulted in only low contribution chimeras that were not capable of transmitting the transgene through the germline. The failure of ten ES cell clones to contribute significantly to chimeras indicated a potential defect in the ES cell clones themselves. For this reason, electroporation was repeated for *Tre2-Hand1*, generating an entirely new set of transgenic ES cell clones.

The new set of ES cell clones proved more successful with germline transmission being obtained for a further two transgenic *Tre2-Hand1* lines, both with a copy number of two. Again, a high number of ES clones were identified as containing the transgene (17/46) and five chimeras were generated. Two chimeras derived from clone 46, one 90% the other 65%, transmitted the transgene through the germline producing transgenic F1 mice, and the 20% chimera derived from clone 26 produced two agouti pups, both of which carried the *Tre2-Hand1* transgene. Transgenic mice from both lines were received and used to expand the colony. Therefore, four transgenic *Tre2-Hand1* lines were generated in total.

2.5 Summary

With the successful derivation of a transactivator strain targeted to the endogenous *Hand1* locus, *Tet-Off-Hand1*, and multiple transgenic *Tre2-Hand1* responder strains, all components were in place for the tet-inducible *Hand1* model. Therefore, in addition to

expanding the colonies, each strain was tested *in vivo* to ensure the expression pattern was correct (see Chapter 4), and were intercrossed to generate compound heterozygote (see Chapter 4) and transheterozygote embryos (see Chapter 5) in order to undertake the loss of function, and gain of function studies respectively, of Hand1 in the developing mouse heart.

However, prior to the *in vivo* studies and parallel to the generation of the mouse strains, the three constructs were tested extensively *in vitro* to ensure correct expression, repression by dox and functional activity.

Chapter 3: Testing the tet-inducible *Hand1* system *in vitro*

3.1 Introduction

3.1.1 *Tet-Off-Hand1*

The Tet-Off transactivator (tTA) is known to recognise and bind to the tet response element, in the absence of dox, and activate expression of a downstream gene of interest (see section 1.4.2). The *Tet-Off-Hand1* transactivator construct was, therefore, tested *in vitro* to demonstrate that it was fully functional i.e. both able to transactivate via TRE and switched off in response to dox. In order to do this, we had to use the P19 embryonic carcinoma cell line since the *Hand1* promoter is active in this cell line, and the *Hand1* promoter drives expression of the tTA in the *Tet-Off-Hand1* construct.

3.1.2 *Tre2-Hand1-EGFP*

Since Hand1 is a transcription factor known to bind DNA (Hollenberg et al., 1995), the *Tre2-Hand1-EGFP* targeting construct was tested *in vitro* to ensure it was transcriptionally functional. It was tested whether the responder translocates appropriately to the nucleus and is able to function as a transcriptional activator, and moreover, whether the EGFP moiety had any adverse effects on Hand1 function. It was also important to confirm that the TRE was responsive to a Tet-Off transactivator resulting in *Hand1-EGFP* expression (mRNA and protein), and that this expression could be 'switched off' by addition of dox.

It is important that the *Tre2-Hand1-EGFP* construct is not 'leaky' in the absence of transactivation so that *Hand1* is both 'switched on' at the correct timepoint, and completely 'switched off' upon the addition of doxycycline. *In vitro* differentiation of ES cells into embryoid bodies provides the closest possible comparison to *in vivo* embryonic differentiation, as embryoid bodies contain representatives of all embryonic cell types, including cardiomyocytes (see section 1.3). Therefore, the expression of the *Tre2-Hand1-EGFP* construct was investigated under conditions of embryonic differentiation.

3.1.3 *Tre2-Hand1*

The transgenic *Tre2-Hand1* construct was also tested *in vitro*. As with the targeting responder construct, *Tre2-Hand1-EGFP*, the transgenic construct must be expressed correctly and responsive to dox, and be able to recognise and bind relevant DNA binding sites and protein partners. Additionally, there must be no leaky expression of the transgene. Again, ES cells containing a stable integration of the *Tre2-Hand1* transgene were differentiated *in vitro* and assayed for appropriate *Hand1* expression.

3.2 Methods

3.2.1 Cell culture

3.2.1.1 P19 cells

P19 cells are a pluripotent murine embryonic carcinoma (EC) cell line that can be maintained in culture either in an undifferentiated state, or induced to differentiate into a variety of cell types such as cardiac and skeletal muscle (McBurney et al., 1982); ATCC Catalogue number: CRL-1825). Cells were cultured in tissue culture plates and maintained as EC cells in αMEM +glutamax supplemented with 7.5% BCS, 2.5% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin and 100 μM β-Mercaptoethanol, at 37°C in a humidified 5% CO_2-air atmosphere. Regular passage, before the cells reached confluency, was carried out by treating the cells with 0.05% trypsin/ 0.02% EDTA for 5 minutes at 37°C until the cells lifted off the plate. The cells were washed in calcium- and magnesium-free Dulbecco's PBS, and then thoroughly resuspended in fresh medium by repeatedly passing through a 10 ml pipette. The cells were re-seeded between 1 in 4 and 1 in 10 dilution.

3.2.1.2 H9c2

H9c2 cells are a rat DB1X heart myoblast cell line (ATCC catalogue number: CRL-1446). H9c2 cells were maintained under standard conditions, as described above for P19, in DMEM + glutamax supplemented with 10% FCS, 100 units/ml penicillin and 100 μg/ml streptomycin.

3.2.1.3 NIH 3T3

NIH 3T3 cells are a murine fibroblast cell line (ATCC catalogue number: CRL-1658) and were maintained under standard conditions in DMEM + glutamax supplemented with 10% BCS, 100 units/ml penicillin and 100 μg/ml streptomycin.

3.2.1.4 293 Tet-Off™

293 Tet-Off™ cells (Clontech catalogue number: 630908) are derived from the parent HEK 293 (human embryonic kidney) cell line and stably express a Tet-Off transactivator. 293 Tet-Off™ cells were cultured under standard conditions in α-MEM + glutamax supplemented with 10% Tet-approved FCS (Clontech), 100 units/ml penicillin and 100 μg/ml streptomycin. Prior to seeding 293 Tet-Off™ cells, plates were coated with 0.1% gelatin to ensure cells adhered appropriately and expanded as colonies.

3.2.1.5 Hand1-null ES cells

Hand1-null murine ES cells (Riley et al., 2000) were maintained under standard conditions in DMEM + glutamax supplemented with 15% ES-qualified FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 1x non-essential amino acids, 1 mM sodium-pyruvate, 100 μM β-Mercaptoethanol and 10^3 units/ml ESGRO™. Prior to seeding ES cells, plates were coated with 0.1% gelatin to ensure cells adhered appropriately and expanded as colonies.

3.2.1.6 Administration of dox

When cells were cultured in the presence of dox, dox was added to the medium, as a solution in dH_2O, to a final concentration of 2 μg/ml.

3.2.2 Transfection

Adherent cells were transfected with plasmid DNA using the Effectene™ Transfection Reagent (Qiagen) according to the manufacturer's protocol, amounts of each transfection component used varied with cell line and culture dish (see Table 3.1).

Briefly, cells were plated 12 hours before transfection, such that they had reached approximately 50% confluency at the time of transfection. DNA-condensation buffer (EC Buffer) and Enhancer was added to the DNA, and mixed by vortexing for 1 second. The DNA-enhancer mix was incubated at room temperature for 5 minutes to allow

condensation of the DNA. Effectene™ was added to the DNA-enhancer mix and vortexed for 10 seconds. The samples were incubated at room temperature for 10 minutes to allow Effectene™:DNA complex formation. During this incubation time, the cells were washed with calcium- and magnesium-free Dulbecco's PBS, and fresh medium was added. Fresh medium was then added to the transfection complexes, mixed by pipetting up and down, and added drop-wise to the cells. Swirling the dish gently attained uniform distribution of the complexes. The dishes were incubated under normal conditions for 48 hours.

Table 3.1. ***Transfection of adherent cells using Effectene™ Transfection Reagent***

Cell line	Dish	DNA (μg)	Enhancer (μl)	EC Buffer (μl)	Effectene (μl)	Medium (ml)
293 Tet-Off™ *Hand1*-null ES	10cm	2	16	300	60	3
	60mm	2	16	200	30	1
	6-well	1	8	100	15	0.8
H9c2 NIH3T3	6-well	0.5	4	50	7.5	0.5

3.2.3 Harvesting cells - Preparation of cell lysates for luciferase and β-Gal assays

Transfected cells were washed with PBS twice. 450 μl of 1X Reporter Lysis Buffer (RLB) was added to each well of cells. The cells were scraped and transferred to an eppendorf. The lysates were then frozen at –70°C and thawed at 37°C, and vortexed for 10 seconds to ensure complete lysis. Cell debris was removed by centrifugation at 16,000g for 2 minutes at 4°C, and collecting the supernatant in fresh eppendorfs.

3.2.4 Luciferase Assay

100 μl of Luciferase Assay Buffer (Promega) was added to 20 μl of cell lysate in a Turner Designs TD-20/20 Luminometer. Luminescence was measured at room temperature for 10 seconds, and recorded as relative light units (RLU).

3.2.5 β-Gal Assay

50 µl 1X RLB and 150 µl 2X β-Gal assay buffer (see Appendix 1) were added to 100 µl cell lysate and vortexed. Samples were incubated at 37°C until the yellow o-nitrophenyl was produced. To stop the reaction, 300 µl of 1M Na_2CO_3 was added and the samples vortexed. The A_{420} was measured for each sample and determined to lie in the linear (quantitative) range between 0.2 and 0.8.

3.2.6 RNA extraction

3.2.6.1 Total RNA extraction

Total RNA was extracted with Trizol LS reagent (Invitrogen) according to manufacturers instructions.

Cells were washed and harvested 48 hours post-transfection in PBS, then resuspended and lysed in Trizol LS reagent. The cells were incubated in Trizol LS reagent for 5 minutes at room temperature to disassociate nucleoprotein complexes. The samples were then extracted in chloroform for 15 minutes and spun at 11,000g at 4°C to separate the upper aqueous phase containing the RNA. RNA was precipitated from the aqueous phase with isopropanol for 10 minutes and spun again as before. The RNA pellet was washed in 75% ethanol then resuspended in 30 µl of DEPC-treated water.

3.2.6.2 Poly(A) RNA extraction

Poly(A) RNA (mRNA) was extracted from cells using the Micro-FastTrack™ 2.0 Kit (Invitrogen), according to the manufacturers instructions.

Cells were harvested and washed twice in DEPC-treated PBS. The cell pellet was freeze/thawed at –70°C, then resuspended and lysed by adding 1 ml of Micro-FastTrack™ 2.0 Lysis Buffer (see Appendix 1). The lysate was incubated at 45°C for 15-20 minutes to allow complete digestion of proteins and ribonucleases. The NaCl concentration of the samples was adjusted to a final concentration of 0.5 M. The lysate was then passed through a 21-gauge needle three or four times to shear any remaining DNA. The lysate was added to an oligo(dT) cellulose column and placed on a rocking platform at room temperature for 20 minutes to one hour. The oligo(dT) cellulose was pelleted and washed three times in Binding Buffer (see Appendix 1). The pellet was then resuspended in Binding Buffer and transferred to a spin column. The spin column

was then washed at least three times with Binding Buffer. A further two washes were done with Low Salt Wash Buffer (see Appendix 1). The mRNA was eluted from the spin column with Elution Buffer (see Appendix 1). The mRNA was precipitated with 20 μg glycogen carrier, 70 mM sodium acetate (pH 5.2) and 100% ethanol, and frozen on dry ice. The mRNA was pelleted and resuspended in 10 μl of Elution Buffer and stored at –70°C.

3.2.7 Northern blotting

Northern blots were carried out using the NorthernMax™ Formaldehyde-Based System from Ambion, according to their protocol, with mRNA extracted from transfected cells. All glassware and electrophoresis equipment was rinsed once with RNase*Zap*™ (Ambion) and twice in DEPC-treated water to remove RNases.

2 μg of sample mRNA was mixed with three volumes of Formaldehyde Load Dye (provided with kit), and ethidium bromide to a final concentration of 10 μg/ml. The samples were denatured for 15 minutes at 65°C in a dry heat block, pulsed, and loaded on a 1% agarose, 1X Denaturing Gel Buffer (provided with kit) (RNase-free) gel. The gel was run in 1X MOPS Gel Running Buffer (provided with kit) at 100 Volts until the bromophenol dye front reached the bottom of the gel. The gel was visualised by exposure to UV light, and photographed alongside a ruler. The RNA was then transferred to positively charged nylon membrane (Hybond™ N+) by the downward capillary transfer method in Transfer Buffer (provided with kit). The RNA was transferred for two hours. Following transfer, the membrane was rinsed in 1X MOPS Gel Running Buffer and baked at 80°C for 20 minutes to cross-link the mRNA. The membrane was then prehybridised at 42°C for 30 minutes to one hour in ULTRAhyb™ (Ambion; pre-heated to 68°C). Prehybridisation and hybridisation was carried out in a Hybaid bottle rotator hybridisation oven. The DNA probe was radiolabelled by the random prime labelling method described under section 2.2.8. The radiolabelled probe was denatured then added immediately to the ULTRAhyb™ and prehybridised blot. Hybridisation was carried out overnight at 42°C. The membrane was then washed with two five minute washes at room temperature with agitation using low stringency wash solution (see Appendix 1), followed by two 15 minute washes at 42°C in the hybridisation oven using high stringency wash solution (see Appendix 1). The blot was then wrapped in Saran Wrap and exposed to film for 15 minutes to overnight, depending on relative signal strength as determined by Geiger counter measurement.

3.2.8 DNase treatment of poly(A) RNA samples prior to RT-PCR

100 ng mRNA was incubated with 1 unit of RQ1 RNase-free DNase (Promega) in 1x RQ1 RNase-free DNase reaction buffer in DEPC-treated water to a final volume of 10 µl for 30 minutes at 37°C. 1 µl of RQ1 DNase Stop Solution was added to terminate the reaction, and the samples incubated at 65°C to inactivate DNase activity.

3.2.9 Reverse transcription-PCR (RT-PCR)

3.2.9.1 Ready-To-Go™ T-primed first strand kit

1-5 µg total RNA from transfected cells was converted to first-strand cDNA using the Ready-To-Go™ T-primed first strand kit (Amersham Biosciences), according to manufacturers instructions. The RNA was diluted into 33 µl DEPC-treated water and incubated at 65°C for five minutes, and then transferred to 37°C for five minutes. The reaction tube was also incubated at 37°C for five minutes. The RNA solution was then added into the reaction tube without mixing and incubated at 37°C for five minutes. It was then mixed and incubated for one hour at 37°C. PCR was performed using conditions described under section 2.2.9 using 1 µl of the cDNA.

3.2.9.2 First-strand cDNA synthesis using random primers for RT-PCR

100 ng DNase treated mRNA, 300 ng random primers (Promega), 1 mM dNTP mix in DEPC-treated water was incubated at 65°C for five minutes and the placed on ice for two minutes. The reaction mixture consisting of 1x first strand buffer (Invitrogen), 5 mM $MgCl_2$, 0.01 M DTT and 40 units RNasin (Promega) was added to each sample and then incubated at 25°C for two minutes. 50 units of SuperScript™ II RT (Invitrogen) were then added to each sample. A 'no RT' control was included for each sample for which reaction conditions were identical except that no SuperScript™ II RT was added. The samples were then incubated for a further 10 minutes at 25°C, followed by 50 minutes at 42°C. The reaction was terminated at 70°C for 15 minutes and then chilled to 4°C. All incubations were carried out using PE Applied Biosystems GeneAmp® PCR System 9700 PCR machine. cDNA samples were stored at –20°C. PCR was carried out as described in section 2.2.9 using 1 µl of the cDNA.

3.2.10 PCR

PCR was carried out using the Ready-To-Go™ beads (Amersham Biosciences), the PCR conditions are described under section 2.2.9. Primers for amplifying *Hand1* bHLH are 340 and 341 (150 bp), primers that amplify across the *Hand1* intron are 340 and 512 (250 bp), primers specific for the *Tre2-Hand1* transgene message are 675 and 676 (400 bp), primers for *EGFP* are 426 and 427 (bp), *B-Actin* 294 and 295 (bp), *tubulin* are 694 and 695 (320 bp) and GAPDH are 513 and 514 (see Appendix 2). Primers specific for the *Tre2-Hand1* transgene (675 and 676) that distinguish between message and DNA product, and were designed using the PRIMER3 software within the San Diego Supercomputer Center (SDSC) Biology Workbench 3.2 website (http://workbench.sdsc.edu/).

3.2.11 Protein extraction – preparation of lysates for western blotting

Lysates were prepared using the standard RIPA cell extract method for western blotting.

Cells were washed twice with PBS. For a 10 cm tissue culture plate, the cells were harvested in 0.5 ml ice-cold RIPA buffer (see Appendix 1). A further 0.2 ml RIPA buffer was added to collect any remaining cells and combined with the first lysate. The lysate was passed through a 21-guage needle two to three times to shear the DNA, and then incubated for 30 minutes in a rotating wheel at 4°C. The lysate was spun down for 20 minutes at 15,000g at 4°C and the supernatant containing the protein transferred to a fresh eppendorf and stored at –70°C. The protein concentration was determined using Bio-Rad Protein Assay reagent, according to manufacturers instructions.

3.2.12 Western blotting

Western blotting was performed using the Bio-Rad Mini-Protean® III apparatus.

Lysate samples (10 g of protein) were mixed with an equal volume of 2x Laemmli buffer (see Appendix 1) and denatured for 5-10 minutes at 100°C. The samples were loaded and run on SDS-PAGE gel (see Appendix 1) in 1x Running buffer (see Appendix 1), at 100-140v until the dye front reached the bottom of the gel. The protein was transferred to Hybond™-C nitrocellulose membrane by wet transfer method in transfer buffer (see Appendix 1), at 200 mA for two hours at 4°C. Proteins on the membrane were visualised by staining in 0.2% Ponceau S. The membrane was then

rinsed in 1x TBS (see Appendix 1) and blocked in Blocking buffer (see Appendix 1) for two hours at room temperature on a rocking platform, followed by brief washing in TBST (see Appendix 1). The primary antibody was diluted in blocking buffer to the required concentration, added to the membrane and incubated overnight at 4°C on a rotating wheel. The membrane was washed six times for 10 minutes with Wash buffer (see Appendix 1) on a rocking platform. The secondary antibody was then diluted to the required concentration in Blocking buffer and incubated with the membrane for one hour at room temperature with gentle rocking. All antibodies together with the relevant dilution and source are listed in Appendix 4. The membrane was then washed again six times for 10 minutes with Wash buffer. A further two, five minute washes were carried out in 1x TBS. The membrane was developed using ECL™ Western blotting detection reagents. A 1:1 mix of the two ECL™ components was freshly made and added to the membrane (0.123 ml/cm^2) for one minute, and then exposed to film for five seconds to 15 minutes.

3.2.13 Selection of stable transfectants – killing curve

Stable clones from cells transfected with a targeting construct plus a TK-hygromycin plasmid were selected with hygromycin 48 hours post-transfection. First, a killing curve was performed with concentrations of hygromycin ranging from 50-200 μg/ml. The optimal concentration was found to be 150 μg/ml and so resistant clones were selected at this concentration. After 14 days, resistant clones were picked into 96-well plates and the cultures expanded in media containing a maintenance concentration of hygromycin (50 μg/ml). Once the stable clones had expanded sufficiently, samples were taken for DNA extraction and aliquots were frozen down. DNA was extracted as described in section 2.2.6. Clones were genotyped by PCR for EGFP (see Appendix 2).

3.2.14 *In vitro* differentiation of ES cells into embryoid bodies

ES cells were maintained in culture as described above. At approximately 80% confluency, cells were trypsinised and resuspended in ES medium minus ESGRO to facilitate differentiation of embryonic cell types. Cells were transferred to 10 cm non-coated bacteriological plates, at low density (~10^6) to avoid the formation of aggregates. Cells were maintained in floating culture for 16 days and fed daily. Floating embryoid bodies were checked for EGFP expression by fluorescence microscopy every two days.

3.2.15 Fluorescence microscopy

Fluorescence microscopy to detect EGFP expression was carried out under UV light with a FITC (fluorescein isothiocyanate) filter, on an Olympus IX70 microscope. The maximum excitation wavelength for EGFP is 489 nm, emission 508 nm. The FITC filter set excitates between 475-490 nm and emits between 503 –530 nm making it a suitable filter with which to visualise EGFP. Nuclei were stained with 5 μg/ml Bis Benzamide (Hoechst 33342: Sigma) in PBS for 10 minutes at room temperature, and then washed twice in PBS.

3.3 Results

3.3.1 *Tet-Off-Hand1*

3.3.1.1 The *Tet-Off-Hand1* construct is able to transactivate the tet-response element (TRE).

It was important to ascertain that the *Tet-Off-Hand1* transactivator construct can recognise and bind the tet response element, and is repressed in the presence of dox. P19 cells are a pluripotent embryonic carcinoma cell line, in which the *Hand1* promoter is active. Therefore, since the transactivator is under the control of the *Hand1* promoter, appropriate transactivator expression is predicted. The *Tre-Tight-Luc* is a tet-responsive luciferase reporter construct that contains modified Tre2 sequences. The Tre-Tight response element consists of seven direct repeats of a 36-bp sequence that contains the 19-bp tet operator sequence (*tetO*), whereas Tre2 consists of seven repeats of a 42-bp sequence containing an 18-bp *tetO* (see section 1.4.2). As a consequence of these modifications, the Tre-Tight response element displays markedly lower levels of background activity than Tre2, as demonstrated by transfections in NIH3T3 cells (Figure 3.1).

P19 cells were transiently co-transfected with a tet-responsive luciferase reporter *Tre-Tight-Luc* (Clontech) and the *Tet-Off-Hand1* transactivator construct, and in the presence or absence of dox. Non-transfected cells and single transfections were included as controls. pCMVβ-Gal was co-transfected for normalisation. Cells were harvested 48 hours post-transfection and luciferase and β-Gal assays were carried out. Relative transcriptional activation was determined by measuring relative luciferase units

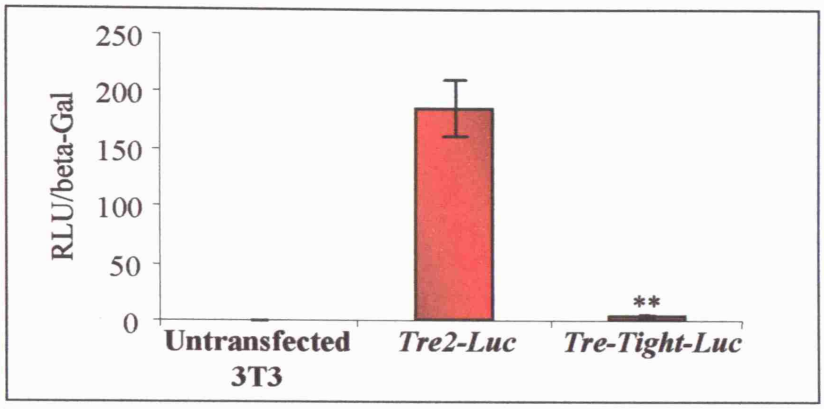


Figure 3.1. Tre-Tight is significantly less 'leaky' than Tre2 *in vitro*. The Tre2 sequence was modified, generating the Tre-Tight tet-response element, in order to reduce the level of background activity in the absence of transactivation by Tet-Off/On transactivators. NIH3T3 cells were transfected with either the *Tre2-Luc* or *Tre-Tight-Luc* reporter in the absence of dox. pCMVβ-Gal was co-expressed for normalisation. Total protein was isolated after 48 hours and luciferase and β-Gal assays were performed using standard protocols. There is over a 40-fold reduction in background activity using the *Tre-Tight-Luc* compared to the *Tre2-Luc*. ** $p<0.02$(Student's t-test; n=3).

(RLU) over the β-Gal A_{420} reading. *Tet-Off-Hand1* was able to transactivate the tet-responsive reporter gene, showing three-fold activation relative to reporter alone (Figure 3.2).

In addition, the *Tet-Off-Hand1* transcriptional response to administration of doxycycline was tested. As expected, reporter activity was reduced to a background level in the presence of dox due to loss of transactivation of *Tre-Tight-Luc* by the Tet-Off transactivator (Figure 3.2).

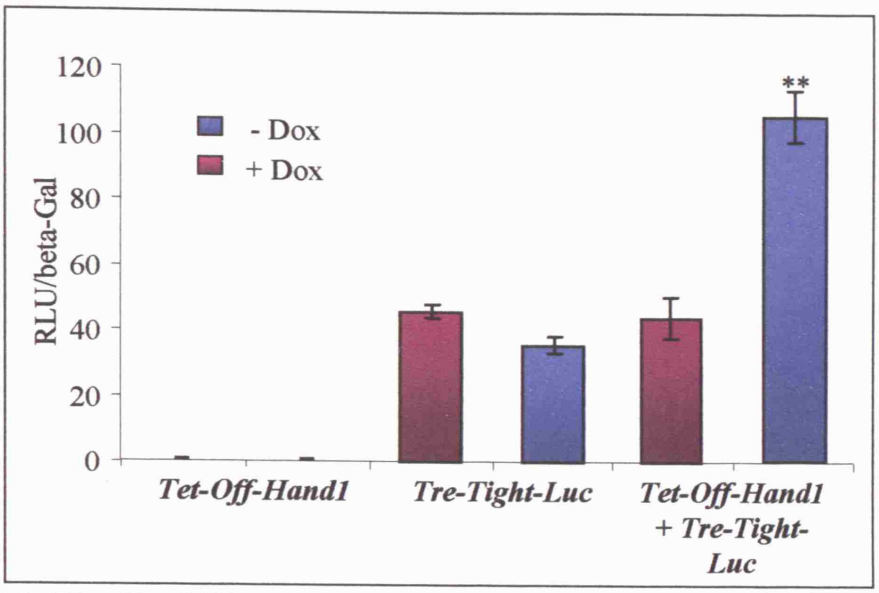


Figure 3.2. The *Tet-Off-Hand1* construct is able to transactivate the tet-response element (TRE). P19 cells were transiently transfected with a tet-responsive luciferase reporter *Tre-Tight-Luc* in the presence or absence of the *Tet-Off-Hand1* transactivator, and in the presence or absence of dox. pCMVβ-Gal was co-expressed for normalisation. Total protein was isolated after 48 hours and luciferase and β-Gal assays were performed using standard protocols. Note (i) the three-fold activation of the reporter by *Tet-Off-Hand1* as compared to reporter alone, and (ii) the background activity in the presence of dox due to loss of transactivation of *Tre-Tight-Luc* by the Tet-Off transactivator. ** p=0.025 (Student's t-test; n=3).

3.3.2 *Tre2-Hand1-EGFP*

3.3.2.1 *Tre2-Hand1-EGFP* is 'switched off' in the presence of doxycycline

Furthermore, it was essential to demonstrate that the expression of *Tre2-Hand1-EGFP* is responsive to doxycycline, i.e. that in the absence of dox, the tTA induces expression of *Tre2-Hand1-EGFP,* and in the presence of dox when the tTA is inactive, there is no 'leaky' expression of *Tre2-Hand1-EGFP.* 293 Tet-Off™ cells were transiently transfected in duplicate with the *Tre2-Hand1-EGFP* construct and were maintained for 72 hours either in the presence (+) or absence (-) of dox, or were given dox 48 hours post-transfection (48+) for the remaining 24 hours. Before harvesting, the cells were visualised by fluorescence microscopy to check for EGFP expression (data not shown).

In the presence of dox, EGFP expression was not observed. In the absence of dox, EGFP was expressed. Under the third set of culture conditions, 48+, EGFP was expressed at 48 hours, prior to administration of dox. At 72 hours, EGFP expression was largely not detected, a few cells exhibited residual EGFP expression most likely due to the inherent stability of EGFP. In order to confirm these observations and also have a definitive check of *Hand1* expression, poly (A) RNA and protein were extracted from the duplicates under each of the culture conditions and used for northern and western analyses.

3.3.2.1.1 Tre2-Hand1-EGFP transcript expression

Poly (A) RNA from the three culture conditions described above were analysed by northern blotting. RNA extracted from *in vitro* differentiated R1 wildtype ES cells harvested at day 10 of differentiation was included as a positive control for *Hand1*, since *Hand1* is expressed at relatively high levels in such *in vitro* differentiated cells at this stage (5). Probes specific for *Hand1*, *EGFP* and *GAPDH* (as a control for mRNA loading) were used. The results of the northern were consistent with the observed effect of dox on *EGFP* expression (Figure 3.3). A *Hand1-EGFP* fused transcript of predicted 1.7 kb in size, as detected by the *Hand1* and *EGFP* probes, was expressed in the absence of dox. In the presence of dox or following 24 hours of dox administered 48 hours post-transfection, the fused transcript was absent.

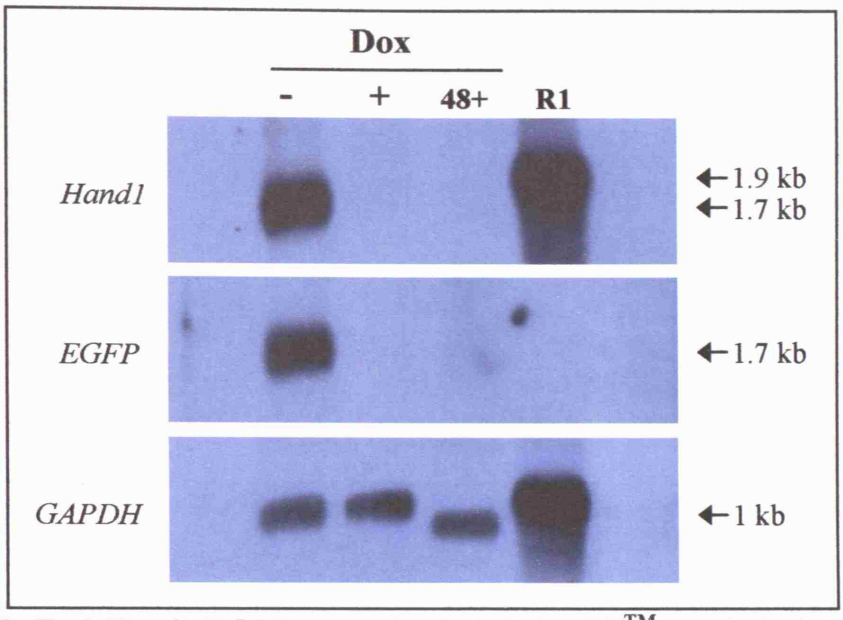


Figure 3.3. *Tre2-Hand1-EGFP* expression in 293 Tet Off™ cells is 'switched off' in the presence of doxycycline. 293 Tet Off™ cells transiently transfected with the *Tre2-Hand1-EGFP* construct (see Figure 2.1) were either maintained for 72 hours in the absence (-) or presence (+) of dox or were given dox 48 hours post transfection (48+) for the remaining 24 hours. Poly(A) RNA was extracted from duplicates under each of the culture conditions and used for northern blot analyses. Poly(A) RNA extracted from R1 wildtype *in vitro* day 10 differentiated ES cells was included as a positive control for *Hand1*. Probes specific for *Hand1*, *EGFP* and *GAPDH* (as a control for mRNA loading) were used. Note the expression of the *Hand1-EGFP* fusion (1.7 kb) in the absence of dox as detected by probes for *Hand1* and *EGFP* and the absence of transcript in the presence of dox or following 24 hours of dox administered 48 hours post-transfection. The 1.9 kb transcript detected exclusively by the *Hand1* probe in the R1 sample represents the full length *Hand1* message with the endogenous 3' UTR.

3.3.2.1.2 Tre2-Hand1-EGFP protein expression

The northern analyses confirmed transcription of the Hand1-EGFP fusion. Western analyses were performed to demonstrate Hand1 and EGFP protein expression. Flag-Hand1, EGFP, a Hand1-EGFP intermediate construct made during the original construct cloning (see section 2.2.1.2), a Hand1-EGFP fusion and untransfected 293 Tet-Off™ cells were included as controls. Protein membranes were probed with anti-C-terminus Hand1 (Santa Cruz) and anti-EGFP (Clontech) antibodies (see Appendix 4). Both Hand1 and EGFP are expressed at the protein level (Hand1 Figure 3.4; EGFP not shown). Note the lack of Hand1-EGFP fusion in the *Tre2-Hand1-EGFP* construct.

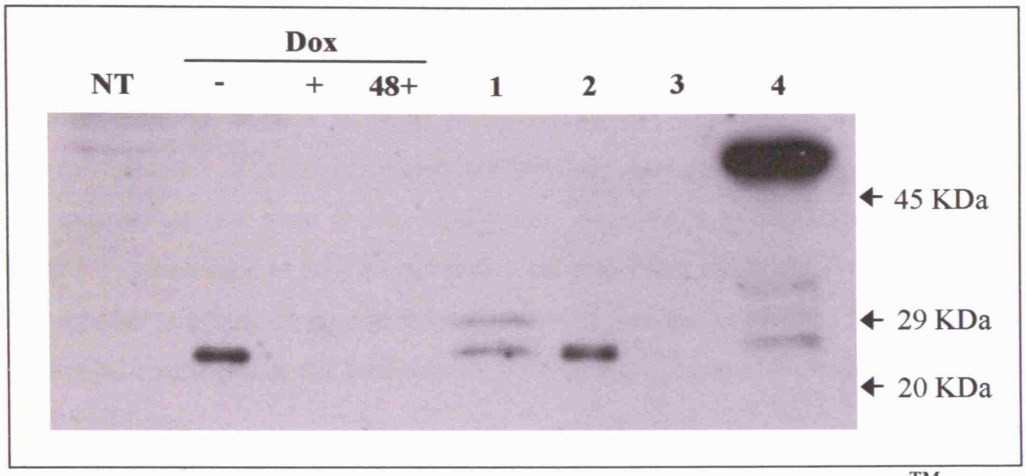


Figure 3.4. *Tre2-Hand1-EGFP* protein expression in 293 Tet Off™ cells is 'switched off' in the presence of doxycycline. 293 Tet Off™ cells were transiently transfected as in Figure 3.3. Whole cell lysates were prepared from duplicates under each of the culture conditions and used for western blot analyses with an antibody to the C-terminus of Hand1 (Santa Cruz). 10 μg of protein were loaded in each lane. Lysates from 293 Tet Off™ cells transiently transfected with Flag-Hand1 (lane 1), a Hand1-EGFP intermediate (lane 2), EGFP-N1 (lane 3; Clontech), a Hand1-EGFP fusion (lane 4) and untransfected 293 Tet Off™ cells (NT) were included as controls. The results confirmed those seen in the northern blot analyses (see Figure 3.3) in that in the absence of dox both Hand1 and EGFP proteins are expressed, but are absent in the presence of dox or following 24 hours of dox administered 48 hours post-transfection. Additionally, the lack of Hand1-EGFP fusion in the *Tre2-Hand1-EGFP* construct was noted. Hand1 protein is 29 KDa , Hand1-EGFP fusion is 50 KDa

3.3.2.2 *Tre2-Hand1-EGFP* is not 'leaky' in absence of transactivation

In order to test whether *Tre2-Hand1-EGFP* expression was 'leaky' in a more physiologically relevant system, *Hand1*-null ES cells were co-transfected with the *Tre2-Hand1-EGFP* construct and a TK-Hyg selection construct. Hygromycin was used for selection of stable clones since the *Hand1*-null cells have been targeted twice already using neomycin and puromycin selection cassettes (Riley et al., 2000), and therefore are resistant to these more standard selection antibiotics. A preliminary killing curve identified 150 μg/ml of hygromycin to be the optimal concentration for selection of stable transformants (not shown). 30 hygromycin resistant clones were picked and expanded in culture. Five clones were further selected for DNA extraction and PCR performed to establish whether *Hand1* and *EGFP* were stably integrated into the

genome. PCR for the house-keeping gene *β-Actin* was also carried out as a positive control. Two out of the five were positive for *Hand1* and *EGFP*. The two stable clones were differentiated *in vitro* to form embryoid bodies. The bodies were observed for EGFP expression by fluorescence microscopy every two days over a 16-day period. No EGFP expression was seen at any time point, from day 0 to day 16 of differentiation (Figure 3.5). The lack of EGFP expression indicated that either the *Tre2-Hand1-EGFP* construct was not 'leaky', or that it had integrated into a silent locus. It was important, therefore, to confirm that *Tre2-Hand1-EGFP* had not integrated into a silent locus in the two ES cell lines.

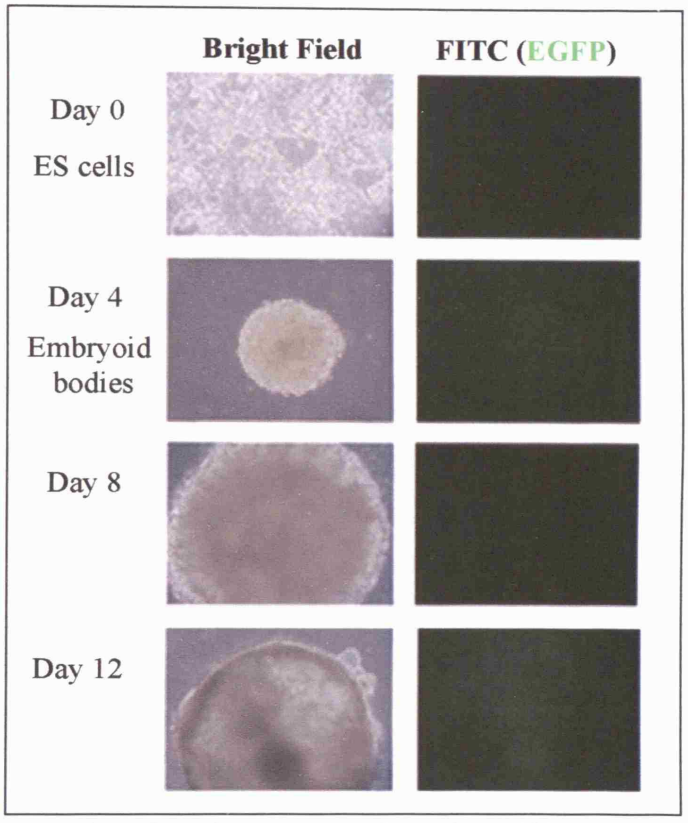


Figure 3.5. *Tre2-Hand1-EGFP* is not 'leaky' during differentiation of embryonic cell types. *Hand1*-null ES cells were co-transfected with the *Tre2-Hand1-EGFP* (see Figure 2.1) and a TK-Hyg selection construct. Stable transformants were selected under hygromycin and expanded in culture. The stable ES cell lines were differentiated *in vitro* to form embyoid bodies. Live ES cells and embryoid bodies were observed under UV with a FITC filter to detect any EGFP expression (right). There was no EGFP expression at any timepoint from day 0 to day 16 of differentiation.

3.3.2.3 Tre2-Hand1-EGFP expression is induced in the stable ES cell lines by a Tet-Off transactivator

The two stable ES cell lines described above were seen not to express EGFP at any time point throughout differentiation. It was important to ensure that the reason for this was that the *Tre2-Hand1-EGFP* construct was not leaky, and not that it had integrated into a silent locus, whereby there would never be any expression observed regardless. Therefore, it was tested that *Tre2-Hand1-EGFP* expression could be induced in the two cell lines.

The two stable *Tre2-Hand1-EGFP* ES cell lines were transiently transfected with pCAGtTA (Kitajima et al., 2002), a Tet-Off transactivator construct under the control of the chick β-actin promoter, a promoter known to be active in ES cells (Chung et al., 2002). Transfected cells were harvested after 48 hours, poly (A) RNA was extracted and used for RT-PCR analysis. PCR was performed for *Hand1* and *tubulin* showing that *Tre2-Hand1-EGFP* expression could be induced in both cell lines (Figure 3.6), and consequently that it had not integrated into a silent locus.

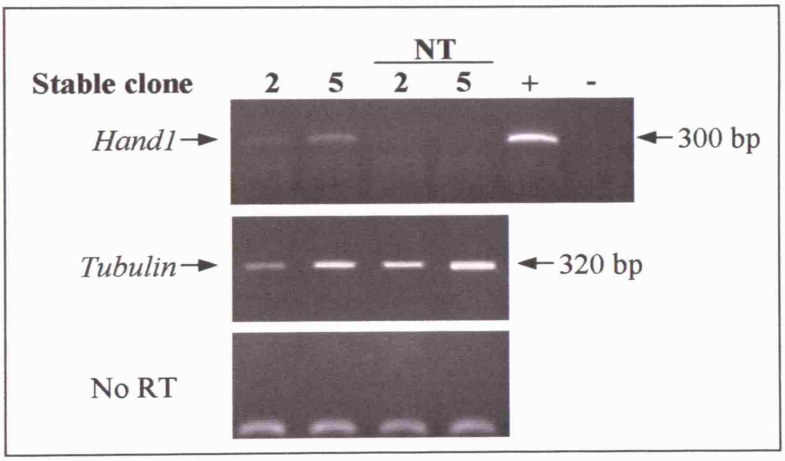


Figure 3.6. A Tet-Off transactivator can induce the *Tre2-Hand1-EGFP* stable lines to express *Hand1*. The *Tre2-Hand1-EGFP* stable ES cell lines (2 and 5) were transiently transfected with pCAGtTA, a Tet-Off transactivator construct under the control of the chick β-Actin promoter (Kitajima et al., 2002) that is known to be active in ES cells. Cells were harvested after 48 hours, poly (A) RNA was extracted and used for RT-PCR analysis. PCR was performed using primers specific for *Hand1* and *tubulin* (see Appendix 2), non-transfected *Tre2-Hand1-EGFP* cells (NT) and a 'no RT' were included as controls. *Hand1* expression was induced in both *Tre2-Hand1-EGFP* stable cell lines.

3.3.2.4 Nuclear localisation of Hand1-EGFP

Since Hand1 is a transcription factor known to bind DNA the *Tre2-Hand1-EGFP* construct was tested to appropriately translocate to the nucleus by transient transfection of the construct into 293 Tet-Off™ cells, alongside a control vector, pEGFP-N1 (Clontech). 293 Tet-Off™ cells (Clontech) are derived from the parent HEK 293 line and stably express a Tet-Off transactivator. Localisation was observed in live cells by fluorescence microscopy to detect EGFP positive cells. The nuclei were visualised by staining with bis-benzamide. Nuclear localisation of Hand1-EGFP is shown in Figure 3.7.

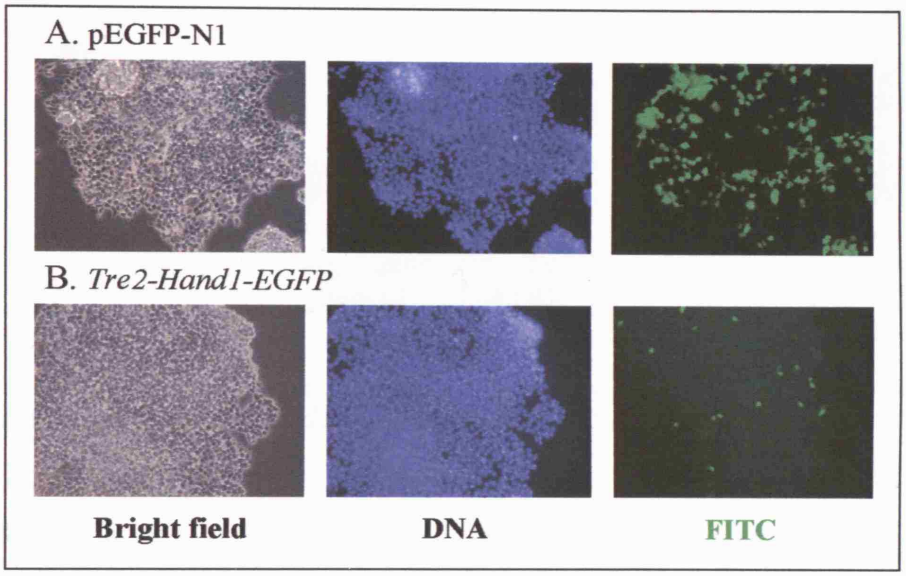


Figure 3.7. *Tre2-Hand1-EGFP* appropriately translocates to the nucleus. 293 Tet Off™ cells transiently transfected with either a control pEGFP-N1 vector (Clontech; A) or the *Tre2-Hand1-EGFP* construct (see Figure 2.1B) in the absence of dox. Live cells were viewed under UV with a FITC filter to detect EGFP positive cells and stained with bis-benzimide to visualise nuclei. Note (i) the expression of EGFP from *Tre2-Hand1-EGFP* under the control of the Tet-Off transactivator in (B) and (ii) the nuclear localisation of the protein, consistent with the localisation of endogenous *Hand1*, as compared to EGFP expression throughout the whole cell in (A).

3.3.2.5 *Tre2-Hand1-EGFP* can activate a 'mock' Hand1 target gene

It was then determined whether *Tre2-Hand1-EGFP* could appropriately transactivate Hand1 target genes. Hand1 has been shown to be a transcriptional activator, binding as

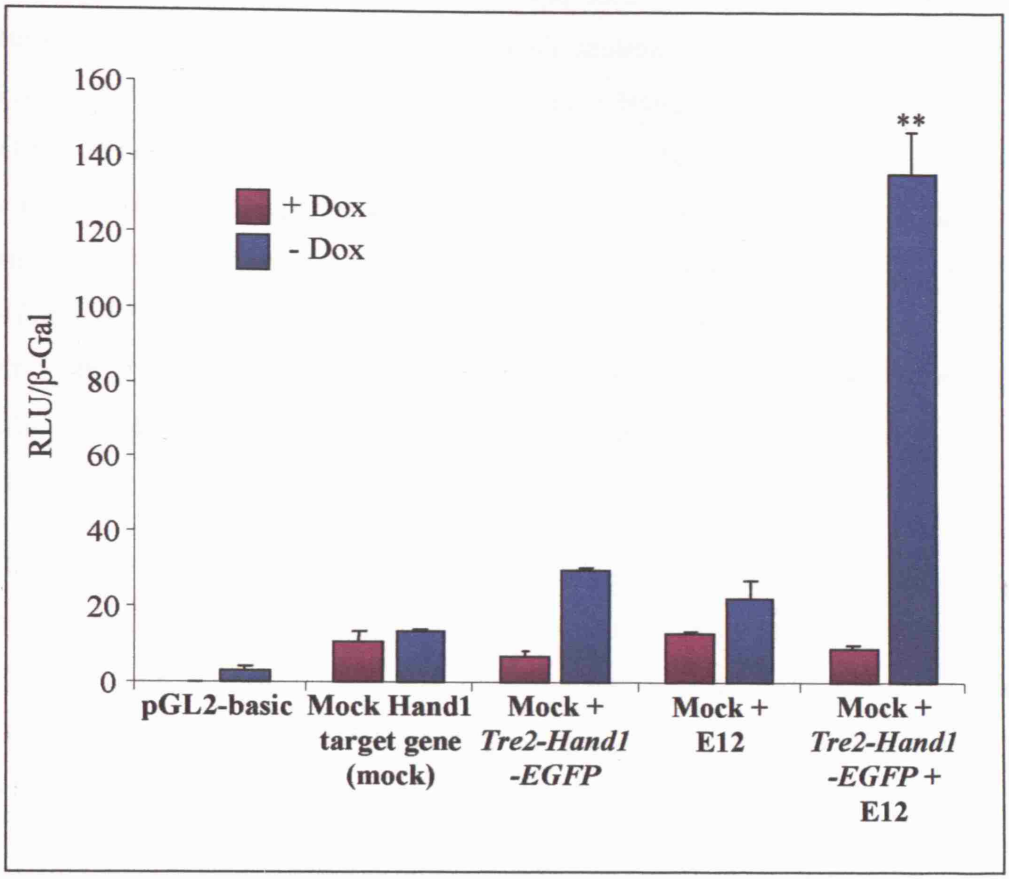


Figure 3.8. The *Tre2-Hand1-EGFP* construct can activate a 'mock' Hand1 target gene. The *Tre2-Hand1-EGFP* construct (see Figure2.1C), in addition to appropriately translocating to the nucleus (see Figure3.7), can activate a 'mock' *Hand1* target gene confirming that the EGFP moiety does not alter Hand1 function *in vitro*. 293 Tet Off™ cells were transiently transfected with a mock Hand1 target gene (mock) plus either the *Tre2-Hand1-EGFP* construct or E-factor (E12) alone or *Tre2-Hand1-EGFP* plus E12 in the presence or absence of dox. pCMVβ-Gal was co-expressed for normalisation. Total protein was isolated after 48 hours and luciferase and β-Gal assays were performed using standard protocols. Note (i) the 14-fold activation of the reporter by *Tre2-Hand1-EGFP* combined with E12 in the absence of dox as compared to reporter alone and (ii) the background activity in the presence of dox due to loss of transactivation of *Tre2-Hand1-EGFP* by the Tet-Off (tetracycline controlled transactivator). ** $p<0.001$ (Student's t-test; n=3).

a heterodimer with class A bHLH E-protein E12 (and E47) to a Thing1 box, a degenerate E-box (Hollenberg et al., 1995)(see section 1.2.1). No direct Hand1 target genes have thus far been characterised, therefore, a Hand1-responsive reporter was used consisting of six concatemerised Thing1 boxes, (CGTCTG), optimised for Hand1/E-factor heterodimer binding (Hollenberg et al., 1995), upstream of an α-cardiac actin minimal promoter driving the luciferase reporter gene (mock Hand1 target gene). The *Tre2-Hand1-EGFP* construct was transiently transfected into 293 Tet-Off™ cells with E12 and the mock Hand1 target gene. Single transfections were also performed as controls. pCMVβ-Gal was co-transfected for normalisation. Luciferase and β-Gal assays were carried out as described in section 3.3.1.1.

Hand1-EGFP was able to transactivate the mock *Hand1* target gene, presumably acting as a homodimer (see section 1.2.1), showing three-fold activation relative to reporter alone (Figure 3.8). Hand1-EGFP in combination with E12 (as a heterodimer (see section 1.2.1)) showed 14-fold activation relative to reporter alone (Figure 3.8).

In addition, the *Tre2-Hand1-EGFP* transcriptional response to administration of doxycycline was tested. As would be predicted, using the inherent 293 Tet-Off™ system, when dox was present in the medium, there were only background levels of transcriptional activation by *Tre2-Hand1-EGFP* (Figure 3.8).

3.3.3 *Tre2-Hand1*

3.3.3.1 *Tre2-Hand1* is 'switched off' in the presence of doxycycline

Again, it was demonstrated that the expression of *Tre2-Hand1* is responsive to dox. In the absence of dox, the transactivator is able to induce expression of *Tre2-Hand1*, with the addition of dox, the transactivator is unable to bind the Tre2 response element preventing expression of *Hand1*. 293 Tet-Off™ cells were transiently transfected in duplicate with the *Tre2-Hand1* construct and were maintained for 72 hours either in the presence (+) or absence (-) of dox, or were given dox 48 hours post-transfection (48+) for the remaining 24 hours. Poly (A) RNA and protein were extracted from the duplicates under each of the culture conditions and used for northern and western analyses.

3.3.3.1.1 Tre2-Hand1 transcript expression

Poly (A) RNA from the three culture conditions described above were analysed by northern blotting. RNA extracted from 293 Tet-Off™ cells transfected with a Flag-Hand1 construct was included as a positive control for *Hand1*. Probes specific for *Hand1* and *GAPDH* (as a control for mRNA loading) were used (Figure 3.9). The transcript (1.7 kb) as detected by the *Hand1* probe, was expressed in the absence of dox. In the presence of dox or following 24 hours of dox administered 48 hours post-transfection, the transcript was absent.

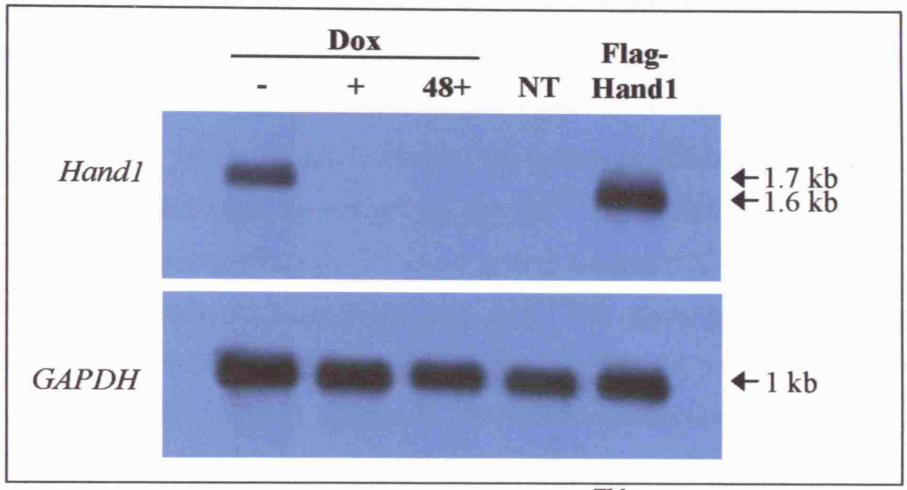


Figure 3.9. *Tre2-Hand1* expression in 293 Tet Off™ cells is 'switched off' in the presence of doxycycline. 293 Tet Off™ cells transiently transfected as in Figure 3.3 with the *Tre2-Hand1* construct (see Figure 2.1). *Flag-Hand1* (1.6 kb) transfected and untransfected (NT) 293 Tet Off™ cells were included as positive and negative controls for *Hand1*. Probes specific for *Hand1* and *GAPDH* were used. Note the expression of the 1.7 kb 'transgenic' *Hand1* transcript in the absence of dox as detected by the *Hand1* probe, and the absence of transcript in the presence of dox or following 24 hours of dox administered 48 hours post-transfection.

3.3.3.1.2 Tre2-Hand1 protein expression

The northern analyses confirmed transcription of the transgenic *Tre2-Hand1* construct. Western analyses were performed to demonstrate Hand1 protein expression using an anti-C-terminus Hand1 antibody (Santa Cruz)(see Appendix 4), and Flag-Hand1 transfected, and untransfected 293 Tet-Off™ cells were included as controls (Figure 3.10). The western analyses demonstrated that Hand1 is expressed at the protein level

in the absence of dox. In the presence of dox or following 24 hours of dox administered 48 hours post-transfection, Hand1 protein was absent.

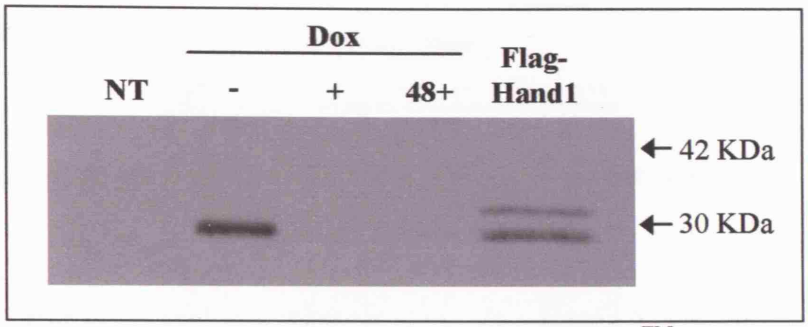


Figure 3.10. *Tre2-Hand1* protein expression in 293 Tet Off™ cells is 'switched off' in the presence of doxycycline. 293 Tet Off™ cells were transiently transfected as in Figure 3.3 with the *Tre2-Hand1* construct (see Figure 2.1). Whole cell protein lysates were prepared from duplicates under each of the culture conditions and used for western blot analyses. *FlagHand1* transfected 293 Tet Off™ cells were included as a positive control for Hand1, untransfected cells as a negative control (NT). An antibody specific for the Hand1 C-terminus (Santa Cruz) was used. The results confirm those seen with the northern blot. Note the expression of 'transgenic' Hand1 protein in the absence of dox, and the absence of Hand1 protein in the presence of dox or following 24 hours of dox administered 48 hours post-transfection. Hand1 protein is 29 KDa

3.3.3.2 *Tre2-Hand1* is not 'leaky' in the absence of transactivation

Again, it was important to ensure that there was no 'leaky' expression from the *Tre2-Hand1* construct in the absence of transactivation by a Tet-Off transactivator in a more physiologically relevant system. Stable ES cell clones containing the *Tre2-Hand1* transgene used to generate mouse strains (see section 2.3.3.1) were obtained and tested *in vitro*. In the absence of transactivation, the *Tre2-Hand1* transgene should remain silent throughout differentiation. The ES cells were differentiated into embryoid bodies *in vitro* up to day eight of differentiation and were harvested every two days. Poly(A) RNA was extracted and northern blotting using a *Hand1* probe, and RT-PCR using primers specific for the *Tre2-Hand1* transgene, endogenous *Hand1* and *tubulin* were performed. Endogenous *Hand1* expression in embryoid bodies is normally detected first at day 4 and peaks at day 10 of differentiation (Smart et al., 2002). The *Tre2-Hand1* ES cell lines expressed endogenous *Hand1* as normal, although expression was not detected at day 4 most likely due to insufficient cDNA as shown by failure to detect a strong level of *tubulin* expression. However, the *Tre2-Hand1* ES cells did not express

the *Tre2-Hand1* transgene at any time point examined, indicating there is no 'leaky' expression, as demonstrated by both northern blotting and RT-PCR (Figure 3.11).

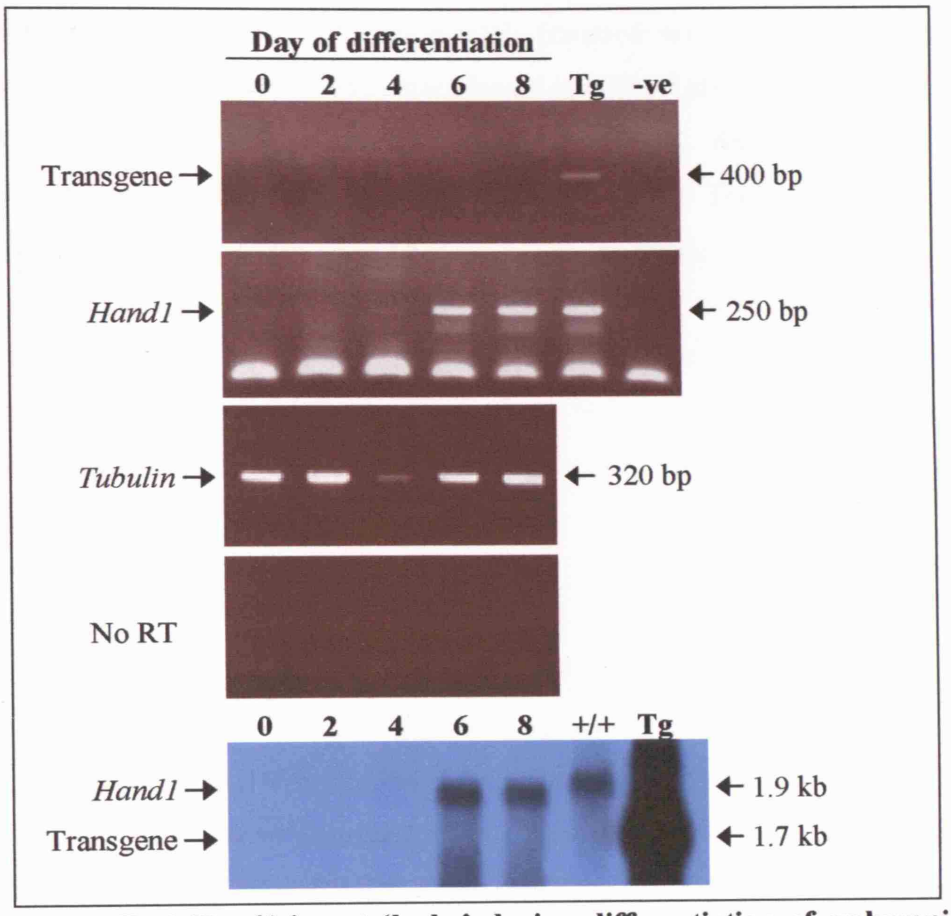


Figure 3.11. *Tre2-Hand1* is not 'leaky' during differentiation of embryonic cell types. *Tre2-Hand1* stable ES cell lines were differentiated into embryoid bodies *in vitro*, harvested at two day intervals until day 8 of differentiation and poly(A) RNA was extracted. (A) RT-PCR was performed using primers specific for the *Tre2-Hand1* message (400 bp), endogenous *Hand1* (250 bp) and *tubulin* (320 bp), using 293 Tet-Off™ cells transfected with *Tre2-Hand1* as a positive control for Hand1. A 'no RT' reaction was included as a control. (B) Northern blotting was performed using a *Hand1* specific probe to detect wildtype *Hand1* (1.9 kb) and *Tre2-Hand1* transgene message (1.7 kb), using a transgene positive control as in A, and E9.5 embryo poly(A) RNA as a control for endogenous Hand1 (+/+). Endogenous *Hand1* expression was detected as normal at day 6 and day 8 of differentiation, whereas the *Tre2-Hand1* transgene was not expressed at any time point examined throughout differentiation.

3.3.3.3 *Tre2-Hand1* can activate a 'mock' Hand1 target gene

As for the *Tre2-Hand1-EGFP* knock-in construct, it was determined whether the *Tre2-Hand1* transgenic construct could appropriately transactivate Hand1 target genes. The *Tre2-Hand1* construct was transiently transfected into 293 Tet-Off™ cells with E12 and the mock Hand1 target gene as described in section 3.3.2.5. As with the *Tre2-Hand1-EGFP* knock-in responder construct (see section 3.3.2.5), *Tre2-Hand1* was seen to transactivate the 'mock' target gene (Figure 3.12). In single transfection, presumably acting as a homodimer, *Tre2-Hand1* activated at a level nearly 1.5-fold above the baseline of reporter alone. When *Tre2-Hand1* was co-transfected with E12, there was synergistic activation of the reporter gene at a level 9-fold above baseline. Additionally, the transcriptional activity of *Tre2-Hand1* was reduced to background levels in response to administration of dox.

3.4 Discussion

Co-transfection of *Tet-Off-Hand1* with a tet-responsive luciferase reporter (*Tre-Tight-Luc*) in P19 cells demonstrated that the *Tet-Off-Hand1* construct is able to transactivate a tet-responsive gene. These co-transfection experiments also demonstrate appropriate repression of the *Tet-Off-Hand1* transactivator by doxycycline.

It was attempted to demonstrate that the *Tet-Off-Hand1* transactivator was able to drive expression of the *Tre2-Hand1-EGFP* responder *in vitro*, by co-transfection of the transactivator and the responder in both NIH3T3 and H9c2 cells. Unfortunately, this was not shown since we were unable to detect either EGFP fluorescence or Hand1-EGFP expression by RT-PCR (not shown). However, this most likely reflects the activity of the *Hand1* promoter/regulatory region, which lies upstream of the Tet-Off transactivator, in the two cell lines used. Co-transfection of *Tet-Off-Hand1* and *Tre-Tight-Luc* demonstrated that there was no expression of the transactivator in these cell lines (not shown), signifying a low level of activity of the regulatory regions in the *Tet-Off-Hand1* construct. These transfections could alternatively have indicated that the *Tet-Off-Hand1* transactivator was unable to transactivate a tet-responsive gene, however the transfections in P19 cells demonstrated that the construct was fully functional *in vitro*.

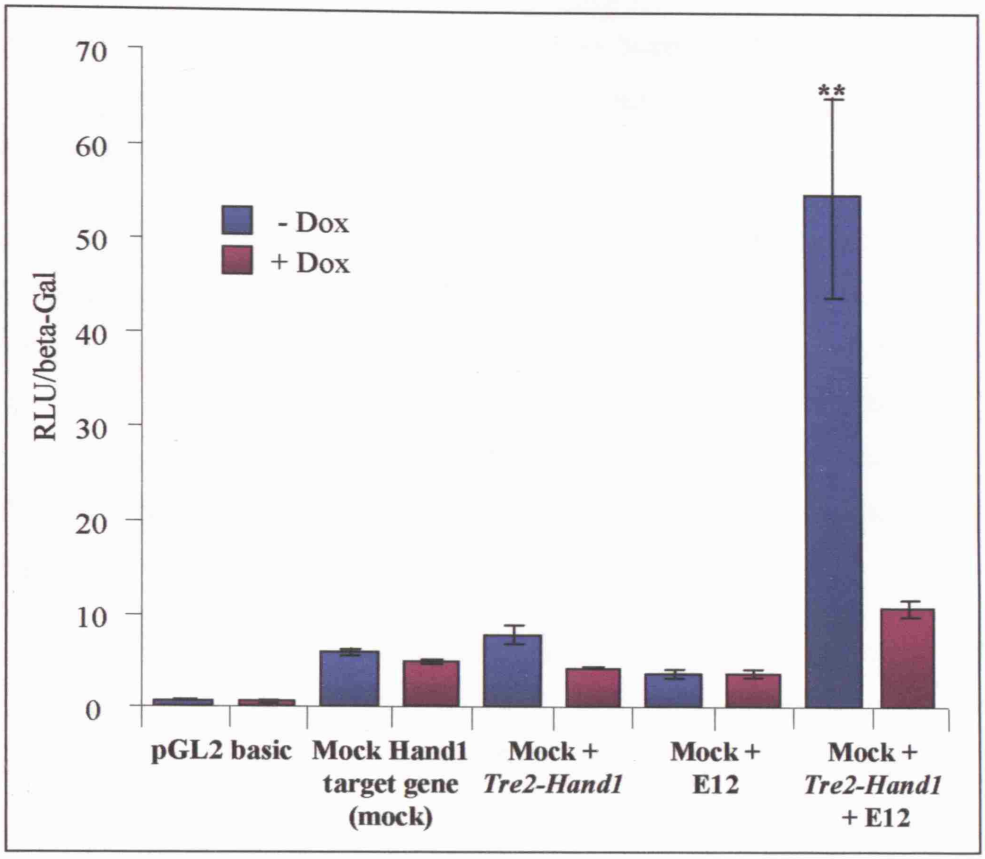


Figure 3.12. The *Tre2-Hand1* construct can activate a 'mock' *Hand1* target gene. 293 Tet Off™ cells were transiently transfected with the mock Hand1 target gene (mock) as in Figure 3.8 with *Tre2-Hand1* in place of *Tre2-Hand1-EGFP*. Note (i) the 5-fold activation of the reporter by *Tre2-Hand1* combined with E12 in the absence of dox as compared to reporter alone and (ii) the background activity in the presence of dox due to loss of transactivation of *Tre2-Hand1* by the Tet-Off (tetracycline controlled) transactivator. ** p=0.05 (Student's t-test; n=3).

The *Tre2-Hand1-EGFP* construct was shown to be expressed appropriately and responsive to dox, not leaky in the absence of transactivation throughout differentiation, and transcriptionally active. The northern and western blotting, along with the EGFP expression, demonstrated that *Tre2-Hand1-EGFP* expressed appropriately and is responsive to dox. Under normal conditions, in the absence of dox, the construct is expressed at both the transcript and protein levels. Addition of dox represses the transactivator and results in the absence of *Tre2-Hand1-EGFP* expression. This indicates that the *Tre2-Hand1-EGFP* construct is responsive to dox, and further demonstrates that the *Tre2-Hand1-EGFP* is not 'leaky' in the absence of

transactivation. Furthermore, expression of the construct can be 'switched off' by the addition of dox. Cells cultured without dox for 48 hours were able to express *Hand1-EGFP*, and then the presence of dox for the remaining 24 hours resulted in complete repression of *Tre2-Hand1-EGFP* expression.

The lack of EGFP expression at any time point throughout differentiation of the *Tre2-Hand1-EGFP* stable ES cell lines demonstrates that the construct is not 'leaky' before and throughout embryonic differentiation as a physiologically relevant model of the *in vivo* situation. The induction of *Tre2-Hand1-EGFP* expression in both the cell lines indicated that the construct had not integrated into a silent locus, and corroborated that the lack of EGFP expression throughout differentiation is by virtue of the fact that *Tre2-Hand1-EGFP* is not leaky.

Nuclear localisation of the Hand1-EGFP fusion protein was observed when the construct was transfected into 293 Tet-Off™ cells. The localisation of the fusion protein was consistent with that of endogenous Hand1 i.e. nuclear, as compared with the control pEGFP-N1, which was localised throughout the whole cell.

Co-transfection of *Tre2-Hand1-EGFP* with a Hand1-responsive luciferase reporter demonstrated that the *Tre2-Hand1-EGFP* construct is able to transactivate a 'mock' *Hand1*-target gene. This also demonstrates that Hand1-EGFP is able to bind DNA recognition sites (Thing1 box) and interact with protein partners (E12), therefore, the EGFP moiety is not affecting Hand1 function *in vitro*. Furthermore, these co-transfection experiments illustrate the repression of *Tre2-Hand1-EGFP* in response to doxycycline, as the presence of dox changes the conformation of the Tet-Off transactivator, inhibiting its ability to bind and activate the responder.

As with the *Tre2-Hand1-EGFP* knock-in responder, the *Tre2-Hand1* transgenic responder construct was shown to be expressed appropriately, transcriptionally active and dox responsive *in vitro*. The northern and western blotting, further demonstrated that *Tre2-Hand1-EGFP* is responsive to doxycycline. Under normal conditions, in the absence of dox, the construct is expressed at both the transcript and protein levels. Addition of dox results in the absence of expression of *Tre2-Hand1*, demonstrating that the *Tre2-Hand1* is responsive to dox and not 'leaky' in the absence of transactivation. Furthermore, as for the *Tre2-Hand1-EGFP* knock-in construct, expression of the transgenic *Tre2-Hand1* construct can be 'switched off' by the addition of dox. Cells

cultured without dox for 48 hours were able to express *Hand1*, supplementing with dox for the remaining 24 hours resulted in complete repression of *Tre2-Hand1* expression. It was further demonstrated that the *Tre2-Hand1* construct was not 'leaky' in the absence of transactivation throughout differentiation *in vitro*. Stable *Tre2-Hand1* ES cell clones were differentiated *in vitro* up to eight days and examined for *Tre2-Hand1* expression by northern blotting and RT-PCR. No *Tre2-Hand1* expression was detected at any time point analysed.

Co-transfection of *Tre2-Hand1* with the mock Hand1 target gene demonstrated that the *Tre2-Hand1* construct is able to transactivate a 'mock' *Hand1*-target gene. This also demonstrates that Hand1 derived from the transgenic cDNA is able to bind DNA recognition sites (Thing1 box) and interact with protein partners (E12) *in vitro*. Furthermore, these co-transfection experiments illustrate the repression of *Tre2-Hand1* in response to doxycycline, via repression of the Tet-Off transactivator.

Chapter 4: Employing the Tet-Off system to generate an inducible knock-out of *Hand1*

4.1 Introduction

The tet-inducible control of *Hand1* expression was tested extensively and shown to function appropriately *in vitro*. This is no guarantee, however, that the system will work successfully *in vivo*. Therefore, upon reception of the *Tet-Off-Hand1* and *Tre2-Hand1* mouse strains it became imperative to analyse the system *in vivo*. Since germline transmission was not successful for the *Tre2-Hand1-EGFP* strain (see section 2.3.2), this strain will not be discussed further.

The employment of a transgenic responder strain means that a bimodal model has been created whereby it is technically possible to study both loss of Hand1 on a *Hand1*-null background, and gain of Hand1 function on a wildtype background. In this chapter, the loss of function, inducible knock-out approach will be discussed. The inducible *Hand1* knock-out model is based upon the fact that the Tet-Off transactivator has been successfully knocked-in to the endogenous *Hand1* locus creating the *Tet-Off-Hand1* strain (see section 2.3.1). This ensures that the Tet-Off transactivator is under the temporal and spatial control of all endogenous *Hand1* regulatory regions. This should ensure that the transactivator is expressed exclusively in *Hand1*-expressing cells within the developing embryo. The transactivator in turn induces expression of *Hand1* from the *Tre2-Hand1* transgene, again exclusively in *Hand1* expressing cells, thereby rescuing the *Hand1*-null phenotype. Tet-inducible expression of *Hand1* can be controlled with dox. Dox can be administered at any time point throughout development to 'switch off' *Hand1* expression, and the effects on the developing heart can be analysed.

In order to study loss of Hand1 function as described above, it is first necessary to determine whether or not the *Hand1*-null phenotype can indeed be rescued with *Hand1* expressed exclusively from the Tet-Off system. This is achieved by combining *Tet-Off-Hand1* and *Tre2-Hand1* on a *Hand1*-null background to generate compound heterozygotes – embryos/mice that carry the *Tet-Off-Hand1* and *Hand1*-null alleles, plus the tet-responsive *Tre2-Hand1* transgene (Figure 4.1). Only in those animals in

which *Hand1* expression comes entirely from the tet system, can *Hand1* expression be switched off with doxycycline at various stages of development and the function of Hand1 in the developing heart analysed.

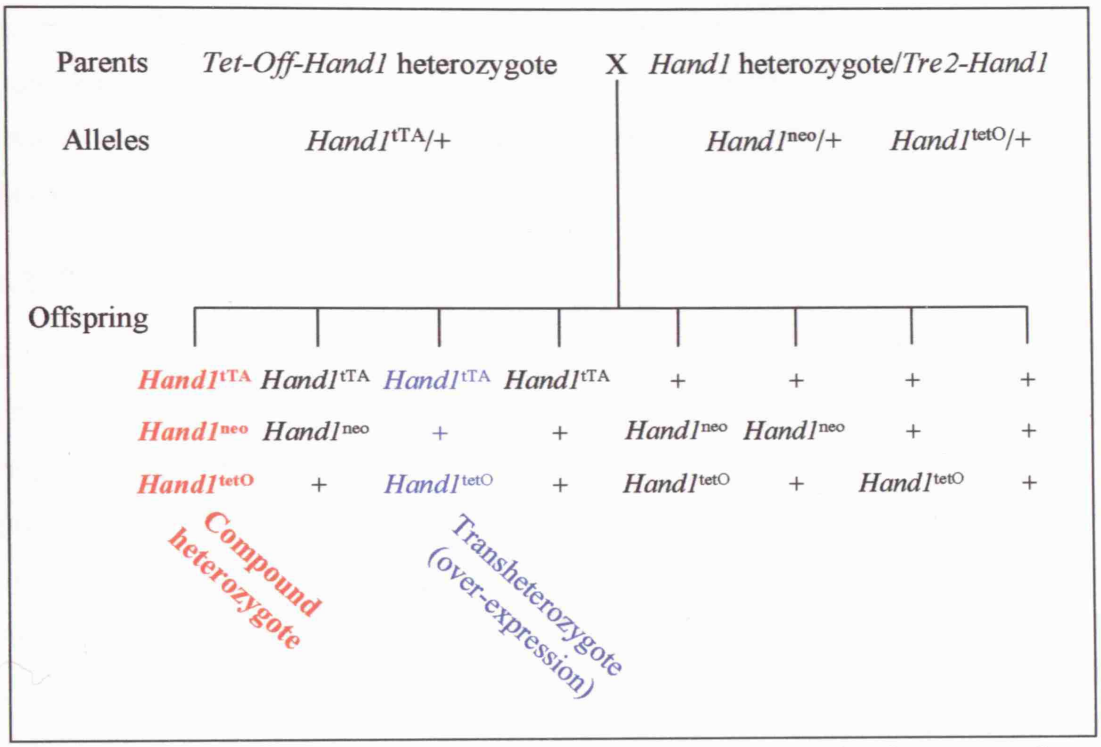


Figure 4.1. Compound heterozygotes have an expected Mendelian frequency of one in eight. In order to rescue the *Hand1*-null phenotype using the Tet-Off system, compound heterozygotes (*Hand1*^{neo}/*Hand1*^{tTA}/*Hand1*^{tetO}) must be generated that have a normal phenotype. Compound heterozygotes are generated by crossing a *Tet-Off-Hand1* heterozygote with a *Hand1* heterozgote that carries the *Tre2-Hand1* transgene. This cross results in eight different genotypes, one of which is the compound heterozygote/rescue genotype, shown in red. Also generated in this cross are the transheterozygote embryos that carry both the targeted Tet-Off transactivator and the transgenic *Tre2-Hand1* responder, shown in blue. These transheterozygote embryos provide the potential gain of function, *Hand1* over-expression model.

Prior to embarking upon the loss of function studies it was necessary to analyse the *Tet-Off-Hand1* strain and the two lines of the *Tre2-Hand1* strain individually to ensure appropriate expression and/or function. The final two *Tre2-Hand1* lines (see section 2.3.4) have only recently been received in house, therefore, studies with these lines have not yet begun and are not discussed further.

4.1.1 *Tet-Off-Hand1*

Since the Tet-Off transactivator has been targeted to the endogenous *Hand1* locus, it was evaluated whether the expression of the transactivator correlated to that of *Hand1*, both temporally and spatially, throughout development. Crossing with a tet-responder/reporter strain assesses the functional activity of the *Tet-Off-Hand1* transactivator strain, where the transactivator should induce expression of the tet-responsive transgene.

4.1.2 *Tre2-Hand1*

The *Tre2-Hand1* transgene should remain silent throughout embryogenesis until appropriate transactivation by the Tet-Off transactivator. The two *Tre2-Hand1* transgenic lines were crossed with the *Tet-Off-Hand1* strain in order to determine expression of the transgene induced by the transactivator during embryonic development. Levels of *Tre2-Hand1* transgene expression were also assessed in comparison to a heterozygous level of *Hand1* expression. The *Tre2-Hand1* line that was shown to express *Hand1* at an appropriate level relative to wildtype was crossed into the *Hand1*-null background, and subsequently into the *Tet-Off-Hand1* background, to attempt rescue of the *Hand1*-null phenotype by generating compound heterozygotes at a predicted frequency of one in eight (Figure 4.1).

4.2 Methods

4.2.1 Mouse maintenance

Tet-Off-Hand1 and two *Tre2-Hand1* lines were generated as described in Chapter 2. *Tet-Hand1* and *Tet-LacZ* lines were a kind gift from T. Mohun, NIMR. For maintenance of the colonies, postnatal mice were tail tipped at around ten days and DNA extracted as described in section 2.2.12.2. *Tet-Off-Hand1* and *Tre2-Hand1* were then genotyped by PCR as described in section 2.2.9, *Tet-Hand1* and *Tet-LacZ* were genotyped using primers 624/625 and 622/623 respectively. Primer sequences are listed in Appendix 2.

4.2.2 Embryo generation

The morning of a vaginal plug was designated E0.5. Embryos were dissected at various developmental stages ranging from E8.0 to E14.5. Embryos collected for poly(A) RNA

or protein extraction were snap frozen immediately in liquid nitrogen and stored until processed further. Embryos collected for X-Gal staining were processed immediately as stated in section 4.2.11 Embryos collected for any other procedure were fixed in 4% formaldehyde/DEPC-treated PBS overnight at room temperature.

4.2.3 Superovulation

Pre-pubescent four and a half week old females were induced to superovulate by injection of gonadotrophins. 5 IU pregnant mare's serum/0.9% NaCl, of which the active ingredient is follicle stimulating hormone, is first injected intraperitoneally in early afternoon. 46 hours later, 5 IU human chorionic gondaotrophin/0.9% NaCl, which mimics luteneising hormone, was injected, also intraperitoneally. The females were then set up to mate, resulting in a plug the following morning, again designated E0.5.

4.2.4 Embryo genotyping

To genotype embryos, the yolk sac was taken and DNA extracted as in section 2.2.12.2. Genotyping was done by PCR using the same primers as for mouse tail tip (section 4.2.1). The PCR conditions were the same as described in section 2.2.9 except 35 cycles were performed, primer sequences are listed in Appendix 2.

4.2.5 DNase treatment and RT-PCR

DNase treatment and RT-PCR were carried out as described in sections 3.2.8-3.2.10. Primers for the Tet-Off transactivator are 357 and 575 (150 bp), for *GAPDH* are 513 and 514 (170 bp), for *tubulin* are 694 and 695 (320 bp). Primers for *Tre2-Hand1* transgene are PrG and PrH (Figure 2.1D) and were designed across the β-globin poly(A) to ensure that the primers were specific for the *Tre2-Hand1* generated 400 bp transgene message, with no cross-reactivity with wildtype *Hand1*. The *Tre2-Hand1* primers also distinguish between DNA (900 bp) and message (400 bp), as part of the β-globin poly(A) sequence is spliced out. For all primer sequences see Appendix 2.

4.2.6 Cloning the transactivator riboprobe for *in situ* hybridisation

An *Eco*R1 + *Bam*H1 1 kb fragment of pTet-Off (Clontech) containing the Tet-Off transactivator was ligated into bluescript ($pKS^{+}tTA$).

4.2.7 *In vitro* transcription of DIG-riboprobe

Detailed information of the riboprobes used can be found in Appendix 5. 10 μg of the vector containing the probe sequence was linearised with the appropriate enzyme in a total volume of 50 μl for two hours at 37°C. 1 μl was run on a 1% agarose gel in order to check linearisation. The volume was made up to 100 μl with DEPC-treated dH_2O and the DNA extracted with phenol/chloroform, the aqueous layer removed and the DNA precipitated with 0.1x volume 3M sodium acetate and 2.5x volumes 100% ethanol at –70°C overnight. The precipitated DNA was spun down for 10 minutes at 16,000g, the pellet washed in 75% ethanol/DEPC-treated dH_2O and resuspended in DEPC-treated TE to a final concentration of 1 μg/ml. The riboprobe was then transcribed from the linearised template. 2 μg template, 1x transcription buffer, 1 mM DIG-NTP mix, 10 mM DTT, 40 units RNasin and the appropriate RNA polymerase (34 units T3; 40 units T7; 28 units SP6) were incubated at 37°C for two hours. The riboprobe was precipitated with 4 mM EDTA, 100 mM LiCl and 100% ethanol up to 100 μl at –70°C overnight. The precipitate was spun down at 12,000g for 10 minutes, rinsed in 70% ethanol/DEPC-treated dH_2O and resuspended in 10-20 μl depending on recovery. 10% of the pre-precipitated and post-precipitated reaction was run on a 1% RNase-free agarose gel to confirm transcription and riboprobe integrity and intensity.

4.2.8 Whole mount *in situ* hybridisation

The whole mount *in situ* protocol is carried out in RNase-free conditions, using DEPC-treated solutions.

E9.5 embryos were dissected out and fixed in 4% formaldehyde/PBT overnight at room temperature, then transferred to 100% methanol. Embryos were stored at –20°C if necessary, for up to one week. The embryos were then rehydrated through 75%, 50% and 25% methanol in PBT for five minutes each, then washed twice in PBT for 10 minutes each. The embryos were then digested with 10 μg/ml ProteinaseK in PBT for 15 minutes at room temperature, rinsed once in PBT and post-fixed for 30 minutes in 4% formaldehyde in PBT and 0.1% gluteraldehyde. Following post-fixing the embryos were washed twice in PBT, for five minutes, and then incubated in hybridisation solution (see Appendix 1) for 10 minutes at room temperature. The embryos were then transferred to fresh hybridisation solution, placed in a 'humid box' and prehybridised overnight at 68°C in a Hybaid oven. The riboprobe was denatured at 95°C for three

minutes and cooled immediately on ice. Embryos were then incubated with the riboprobe at an approximate concentration of 400 ng/ml in fresh hybridisation solution, overnight at 68°C in a Hybaid oven. After hybridisation with the riboprobe, the embryos were rinsed twice for five minutes, and washed three times for 30 minutes in hybridisation solution, pre-warmed to 68°C. A further wash at 68°C was carried out using pre-warmed 1:1 hybridisation solution:TBST (see Appendix 1) for 20 minutes. Next, the embryos were rinsed three times for five minutes and washed three times for 30 minutes in TBST at room temperature with rocking. The embryos were subsequently blocked in TBST with 10% sheep serum and 1% BSA (block) for a minimum of three hours at room temperature, with rocking. Then, the embryos were incubated with anti-DIGoxigenin-AP Fab fragments (Roche) at 1:2000, in block, overnight at 4°C, on a rocking platform. Following hybridisation with the antibody, the embryos were rinsed three times for five minutes and washed three times for one hour in TBST and washed twice for 10 minutes in NTMT (see Appendix 1), all at room temperature with gentle rocking. After washing, the embryos were developed in NBT/BCIP solution (one tablet in 10 ml dH_2O; Roche) in the dark at room temperature on a rocking platform. The embryos were checked in the first instance after 15 minutes, and thereafter as deemed necessary, leaving the signal to develop until specific staining is seen to the required intensity. This was usually between two and four hours, depending on the specificity of the riboprobe used. Once the signal strength was sufficient, the embryos were transferred to TBST and washed twice for 10 minutes, then fixed overnight at room temperature in 4% formaldehyde/PBT. Embryos were stored in PBS at 4°C until they were visualised using a Nikon SMZ-U microscope, and photographed using a FujiFilm FinePix S2 Pro digital camera and Hyper-Utility Software HS-S2. In order to intensify the signal, embryos were dehydrated through a methanol/PBT series, five minutes each in 25%, 50%, 75% and twice in 100%, and then rehydrated through the same series back to PBS. The embryos were then photographed as described above.

4.2.9 Tissue processing, wax embedding and sectioning

Processing and wax embedding of embryos was carried out as described by Moorman et al.,(2001).

Following whole mount *in situ* hybridisation the embryos were embedded in wax for sectioning. The embryos were first dehydrated through a graded ethanol series, two

hours in each 50%, 70%, 80%, 90% and 96% ethanol/DEPC-treated dH_2O, followed by two one hour incubations in 100% ethanol. The embryos were then cleared in butanol overnight. Next, the embryos were transferred to a 1:1 mix of butanol:pastillated fibrowax for 30 minutes at 60°C. The embryos were then incubated in wax at 60°C for the entire day and overnight, with three changes of wax. Next, the embryos were placed in a mould, orientated with a warm needle and left to set. The embedded embryos were stored at 4°C until ready for sectioning. Sections were cut at 10 μM thickness on a Microm HM 330 microtome, and carefully picked up in series using TESPA coated slides. The embryo sections were dried on a flat bed dryer for 30-60 minutes at 50°C and stored prior to further processing.

4.2.10 Eosin staining of embryo sections

Slides were de-waxed in 100% Histoclear twice for five minutes then re-hydrated through 100% ethanol twice, then 95%, 80% and 70% once each. The slides were then stained in 0.5 % eosin (aqueous) for one minute and washed in dH_2O. The slides were air-dried, usually overnight, and then mounted with coverslips using DPX mountant. Sections were visualised using a Zeiss Axioplan2 microscope and were photographed using Zeiss Axiophot2 and Prog Res 3012 software (Kontron Elektronik).

4.2.11 X-Gal staining

E9.5 embryos were dissected out in ice-cold PBS and then fixed for 10 minutes in 2% formaldehyde/2 mM $MgCl_2$/PBS at room temperature. The embryos were then washed twice in 2 mM $MgCl_2$ in PBS to remove all traces of formaldehyde. Next, the embryos were transferred to X-Gal staining solution consisting of 20 mM $K_3Fe(CN)_6$, 20 mM $K_4Fe(CN)_6$-$3H_2O$, 2 mM $MgCl_2$, 0.01% DOC, 0.02% NP-40 and 1 mg/ml X-Gal in PBS, and then incubated in the dark at 37°C in a hybaid oven overnight.

4.2.12 Poly(A) RNA extraction, northern blotting and densitometry

Poly(A) RNA was extracted as described in section 3.2.6.2. Northern blotting and densitometry were carried out as described in section 3.2.7 and section 2.2.5 respectively using a *Hand1* probe. However, due to high background being present in the lanes, hybridisation and washing temperature were slightly elevated to 45°C, four additional washes of one hour each were carried out and the amount of probe used was reduced by half. For northern blotting on E9.5 embryos the entire sample of poly(A) RNA was loaded, which was an average of 1 μg.

4.1.13 DNA extraction from embryos and Southern blotting

DNA was extracted from embryos using the method described in section 2.2.6. Southern blotting was performed as described in section 2.2.7. Embryo DNA was digested with *Bgl*II (see Figure 2.1) for 5' probe hybridisations. A 2 kb *Eco*R1-*Kpn*1 probe (see Figure 2.1A) was used flanking the 5' end of the constructs.

4.2.14 Total RNA extraction from embryos and quantitative real-time PCR analysis

Total RNA was extracted using Trizol according to the manufacturer's protocol (see section 3.2.6.1), DNase treated (see section 3.2.8) and first strand cDNA synthesised using Superscript II RT (Invitrogen) as described in section 3.2.9.2. Quantitative real-time PCR was performed by Laurent Dupays, NIMR, on an ABI 7000 Sequence Detector (Applied Biosystems) using SYBR green (Absolute QPCR SYBR Green Mix, ABgene). Primer sequences for *Hand1* can be found in Appendix 3. The primer set had a calculated annealing temperature of 58°C and a validation experiment was performed, according to manufacturer's instructions, to ensure the efficiency of the target (*Hand1*) amplification and the efficiency of the reference (*GAPDH*) amplification were approximately equal. Amplification specificity was checked using the dissociation curve following the manufacturer's instructions. Results were analysed using Real Quant Software (Applied Biosystems). Quantification was performed using the comparative C_T method ($\Delta\Delta C_T$) and fold change was calculated using the formula $2^{-\Delta\Delta C}{}_T$ (Applied Biosystems Guide to Performing Relative Quantitation of Gene Expression using Real-Time Quantitative PCR).

4.3 Results

4.3.1 *Tet-Off-Hand1*

4.3.1.1 The Tet-Off transactivator is expressed in *Tet-Off-Hand1* embryos

In order to determine that the transactivator was expressed at appropriate developmental stages, *Tet-Off-Hand1* heterozygote mice were crossed and embryos dissected at E8.0, E8.5, E9.5 and E10.5. Entire litters were pooled for each developmental stage, regardless of genotype. Poly(A) RNA was extracted and RT-PCR was performed for

the Tet-Off transactivator and *GAPDH* as a control. Tet-Off transactivator expression was demonstrated at the transcript level for these four stages of embryonic development (Figure 4.2).

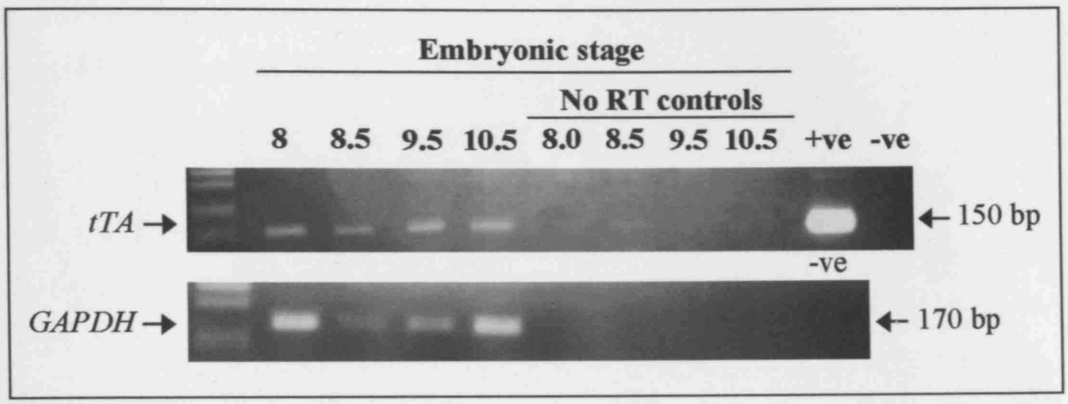


Figure 4.2. The Tet-Off transactivator is expressed in *Tet-Off-Hand1* embryos. *Tet-Off-Hand1* heterozygote mice were crossed and embryos were dissected at E8.0, E8.5, E9.5 and E10.5. Embryos were pooled for each stage regardless of genotype, poly(A) RNA was extracted and RT-PCR performed using primers specific for the Tet-Off transactivator (150 bp), and *GAPDH* (170 bp) as a control. The Tet-Off transactivator expression was detected at all four stages of development.

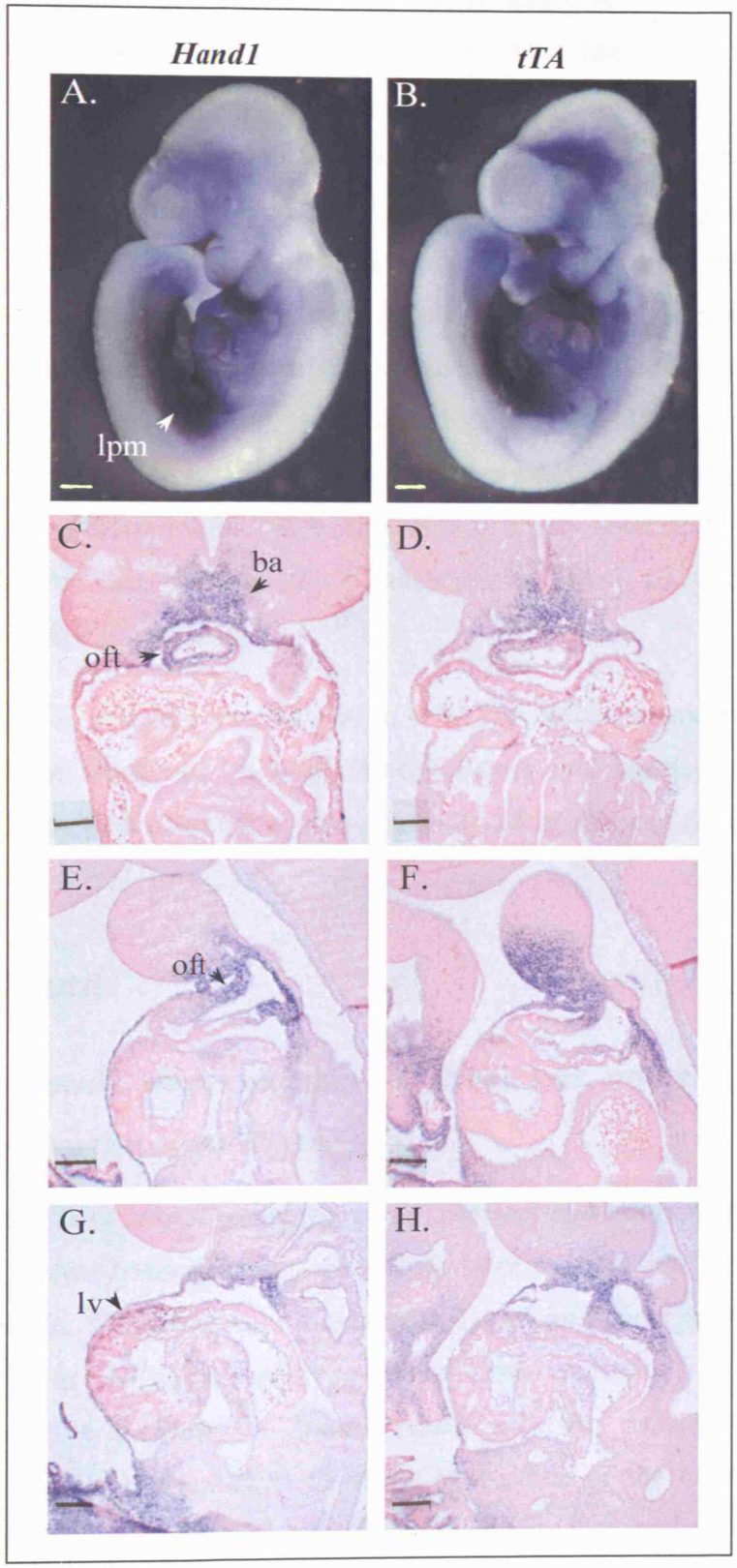
4.3.1.2 The Tet-Off transactivator is co-expressed with *Hand1* in the developing embryo

The pattern of expression of the transactivator in *Tet-Off-Hand1* embryos was examined by whole mount *in situ* hybridisation on E9.5 embryos, to ensure that it was expressed in an analogous pattern to that of wildtype *Hand1*. Following whole mount *in situ* hybridisation, the embryos were embedded in wax and sectioned to examine the transactivator expression pattern more closely. A Tet-Off transactivator probe was generated for this analysis.

In accordance with *Hand1* expression at E9.5, the transactivator was shown to be expressed quite strikingly in the outflow tract (Figure 4.3), and more weakly in the outer curvature of the presumptive left ventricle (Figure 4.3). In addition to being expressed in the heart, it was also seen at the outer margins of the branchial arches and in the lateral plate mesoderm, both of which are areas that are known to express *Hand1*.

Figure 4.3. The Tet-Off Transactivator is co-expressed with *Hand1* at E9.5. *In situ* hybridisation to *Hand1* (A, C, E, G) and Tet-Off transactivtor transcripts (B, D, F, H) on *Tet-Off-Hand1* heterozygote embryos at E9.5. Whole mount left lateral view (A, B), and frontal (C, D) and sagittal (E-H) sections through E9.5 *Tet-Off-Hand1* heterozygote embryos following whole mount *in situ* hybridisation for *Hand1* and Tet-Off transactivator transcripts and counter-staining with eosin. *Hand1* and the Tet-Off transactivator are co-expressed in the developing heart within the outflow tract (oft) and the outer curvature of the presumptive left ventricle (lv), and also in the lateral plate mesoderm (lpm) and branchial arches (ba).

A,B Scale bars approximately 200 μM C-H Scale bars approximately 100 μM


Hand1
tTA
A.
B.
lpm
C.
ba
oft
D.
E.
oft
F.
G.
lv
H.

4.3.1.3 *In situ* hybridisation reveals mosaic expression of the Tet-Off transactivator

Although the Tet-Off transactivator was shown to be expressed in the same regions as *Hand1* in *Tet-Off-Hand1* embryos, it also became obvious that there is variation in the expression pattern of the transactivator (Figure 4.4). In some embryos, expression of the transactivator is strong throughout the distal portion of the outflow tract, particularly the region closest to the body wall, and is expressed more weakly in the ventricle (Figure 4.4A, B). Transactivator expression is also detected in other regions of *Hand1* expression including the lateral plate mesoderm and the branchial arches (Figure 4.4). Some embryos display the same pattern of expression as described above but the overall level of transactivator expression is reduced (Figure 4.4C, D). In other embryos expression may only be detected in the distal outflow tract, at a greatly restricted level (Figure 4.4E-F). Furthermore, in some cases, it was barely detectable anywhere in the embryo (not shown). In the majority of embryos analysed, the transactivator was expressed at a relatively low level.

In situ hybridisation was also carried out on wildtype embryos, and further using the transactivator sense riboprobe on both *Tet-Off-Hand1* and wildtype embryos. The pattern of expression described above was not observed in either of these control groups (not shown).

4.3.2 *Tre2-Hand1*

4.3.2.1 *Tre2-Hand1* copy number 12 line, but not copy number 3, expresses the *Hand1* transgene

To ensure that the *Tre2-Hand1* transgene was expressed, and not randomly inserted into a silent locus, the two transgenic responder lines were crossed with the *Tet-Off-Hand1* transactivator strain. Embryos were dissected at E12.5 and genotyped. Poly(A) RNA was extracted from six single embryos (three from each line) that genotyped as transheterozygotes, with both the transactivator and the transgene. RT-PCR and northern blotting were performed to illustrate expression of the transgene. The copy number 12 line (*Tre2-Hand1*(CN12)) embryos were shown to express the transgene by both RT-PCR and northern, but the copy number 3 (*Tre2-Hand1*(CN3)) embryos failed to express the transgene (Figure 4.5).

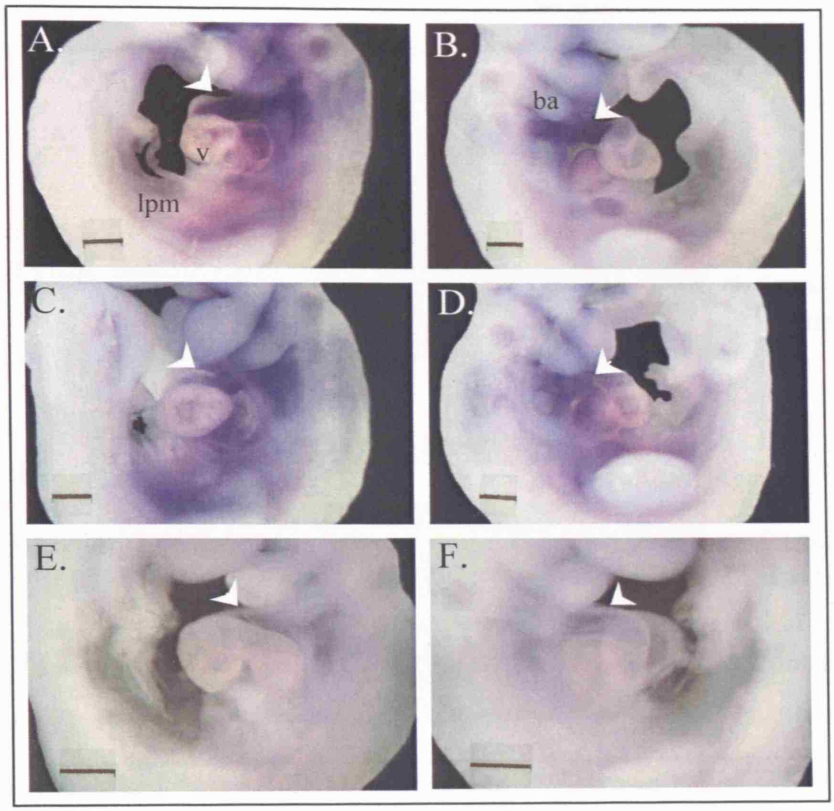


Figure 4.4. *In situ* hybridisation reveals mosaic expression of the Tet-Off transactivator. Whole embryo left lateral view (A, C, E) and right lateral view (B, D, F). Whole mount *in situ* hybridisation to Tet-Off transactivtor transcripts on *Tet-Off-Hand1* heterozygote embryos at E9.5. The transactivator is expressed in the developing heart and is strongest in the distal portion of the outflow tract (white arrows). (C-D) Expression pattern of the transactivator is similar to (A-B) but at a lower level, particularly in the ventricle (C). (E-F) Expression of the transactivator is weak, and only detected in the distal outflow tract (arrows). Scale bars approximately 200 μM

One *Tre2-Hand1*(CN12) embryo did not appear to express the transgene by RT-PCR or northern blotting (Figure 4.5A, B; lane 2). In higher exposures however, a faint trace of transgene message could be seen (not shown). The *tubulin* control indicates that the cDNA levels and presumably therefore poly(A) RNA levels, for this embryo were low. This would affect the efficiency of the PCR reaction and probe hybridisation and could explain the very low level of transgene message.

A further three *Tre2-Hand1*(CN3) transheterozygote embryos were tested for transgene expression by RT-PCR and northern blotting to ensure that this transgenic line definitely did not express the transgene. As expected, it was shown that none of the three embryos expressed the *Tre2-Hand1* transgene (not shown).

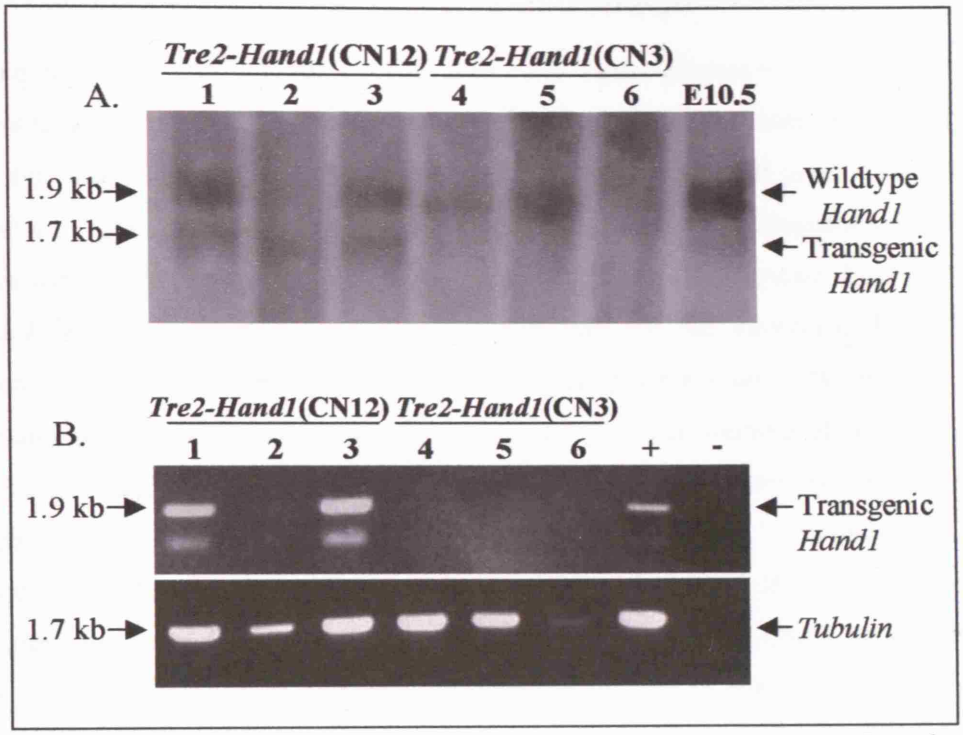


Figure 4.5. *Tre2-Hand1*(CN12) expresses the transgene. E12.5 embryos were generated by crossing *Tet-Off-Hand1* heterozygote and *Tre2-Hand1* transgenic mice. Poly (A) RNA was extracted from three transheterozygote embryos from each *Tre2-Hand1* strain; *Tre2-Hand1*(CN12) lanes 1-3, *Tre2-Hand1*(CN3) lanes 4-6. (A) Northern blot analysis was performed using a *Hand1* probe (see section 3.3.2.1.1), expected transcript sizes are 1.9 kb for wildtype *Hand1* and 1.7 kb for the *Tre2-Hand1* transgene. E10.5 wildtype embryo poly (A) RNA was included as a positive control. (B) Additionally, RT-PCR was performed using primers specific for the transgene message (see section 3.2.10) expecting a 400 bp PCR product, 293 Tet-Off™ cells transfected with *Tre2-Hand1* were included as a positive control and PCR for *tubulin* was performed as a control for cDNA loading. *Tre2-Hand1*(CN12) strain expresses the transgenic *Hand1* message as shown by both northern blotting and RT-PCR (lanes 1 and 3), however *Tre2-Hand1*(CN3) does not (lanes 4 and 5).

4.3.2.2 *Tet-Off-Hand1* crossed with *Tre2-Hand1*(CN12)/*Hand1* heterozygote failed to rescue the *Hand1*-null phenotype

Since only the *Tre2-Hand1*(CN12) responder strain expressed the transgene, it was crossed into the *Hand1* heterozygote background, and subsequently crossed with the *Tet-Off-Hand1* strain with the aim of rescuing the *Hand1*-null phenotype. This cross results in eight possible genotypes, one of which is null for endogenous *Hand1* but has both the transactivator and the transgene (compound heterozygote; Figure 4.1) where any *Hand1* expression is generated exclusively by the integrated Tet-Off system. Because of the low probability (1 in 8) of generating rescued embryos in each litter, females were superovulated in order to increase the total number of embryos generated per litter, and consequently increase the number of potentially rescued embryos. Embryos were dissected at E8.5 and genotyped by PCR. E8.5 was chosen as a starting point since the *Hand1*-null phenotype is clearly evident at this stage. Therefore, it would also be evident whether the *Hand1*-null phenotype could be rescued in the compound heterozygotes. Litter sizes ranged from 7 to 13 embryos, which suggests the superovulation procedure was not optimal. Only one E8.5 embryo in 30 genotyped as compound heterozygote (Table 4.1), and this embryo had the classical *Hand1*-null phenotype (Figure 4.6).

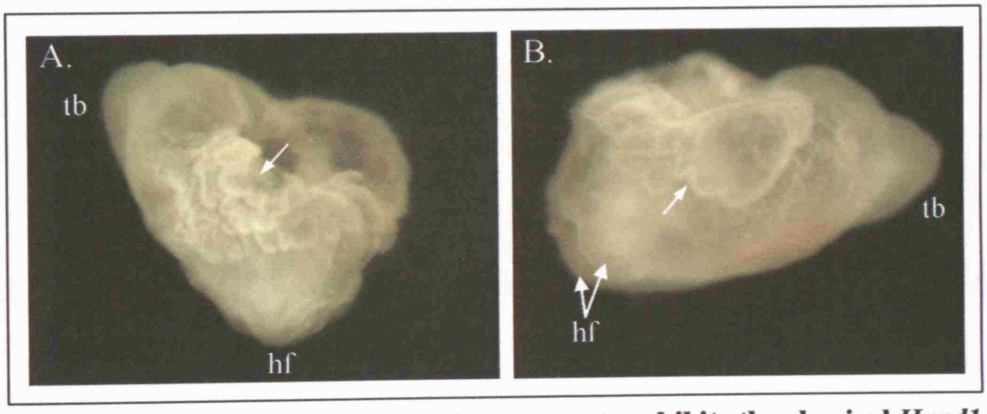


Figure 4.6. A *Tre2-Hand1* compound heterozygote exhibits the classical *Hand1*-null phenotype. (A) Dorsal view and (B) lateral view of a *Tre2-Hand1* compound heterozygote at E9.5. The embryo has arrested at around E7.5 due to extra-embryonic defects, notably haemorrhaging of the yolk sac (arrows). hf, head folds; tb, tail bud.

In addition to embryos, live born litters were analysed. DNA was extracted from tail tips and both PCR and Southern blotting performed to confirm the genotype. Southern

blotting was done, further to the PCR analysis, because there was a concern that it may not be possible to entirely exclude the wildtype band from a PCR reaction due to contamination, and that a *Hand1*-null result may be missed. The Southern blotting corroborated the PCR results in each case (not shown). Unfortunately, no compound heterozygotes were identified in 107 animals (Table 4.1).

Tet-Off-Hand1/Tre2-Hand1(CN12) transheterozygotes were also crossed with each other and with *Tet-Off-Hand1* heterozygotes in an attempt to increase the likelihood of obtaining compound heterozygotes by both reducing the number of possible genotypes, also perhaps by increasing the degree of expression of the transactivator and/or the transgene to a suitable level (multiple loci). Embryos were dissected at E12.5 in order to ensure sufficient amounts of DNA were recovered from each embryo. DNA was extracted from the embryos, genotyped by PCR and Southern blotting was performed on selected embryos in order to verify the PCR results, which it did unequivocally (not shown). None of the 43 embryos recovered at E12.5 were compound heterozygotes (Table 4.1).

Table 4.1. ***Proportion of compound heterozygotes generated***

	***Hand1*^{neo}/*Hand1*^{tTA}/ *Hand1*^{tetO} (rescue)**	**Total**	**Expected frequency**
E8.5	1	30	4
E12.5	0	43	11
Post-natal	0	107	15

4.3.2.3 *Tre2-Hand1*(CN12) expression levels are not sufficient to rescue the *Hand1*-null phenotype

It became clear that despite the fact that the system appeared to be working in terms of expression (the transactivator was being expressed and was inducing expression of the transgene), it was not going to be possible to rescue the *Hand1*-null phenotype using the respective transactivator and responder lines described above. This could be due to expression levels of the transgene being too low to be sufficient to rescue the *Hand1*-null phenotype.

Four crosses were set up between *Tet-Off-Hand1* heterozygotes and *Tre2-Hand1* transgenic mice, embryos were dissected at E12.5, genotyped and poly (A) RNA was extracted. Northern blotting was performed using a *Hand1* probe for selected

transheterozygote embryos, including a control that carried only the *Tre2-Hand1* transgene. Again, the expected message sizes were 1.9 kb for wildtype *Hand1* and 1.7

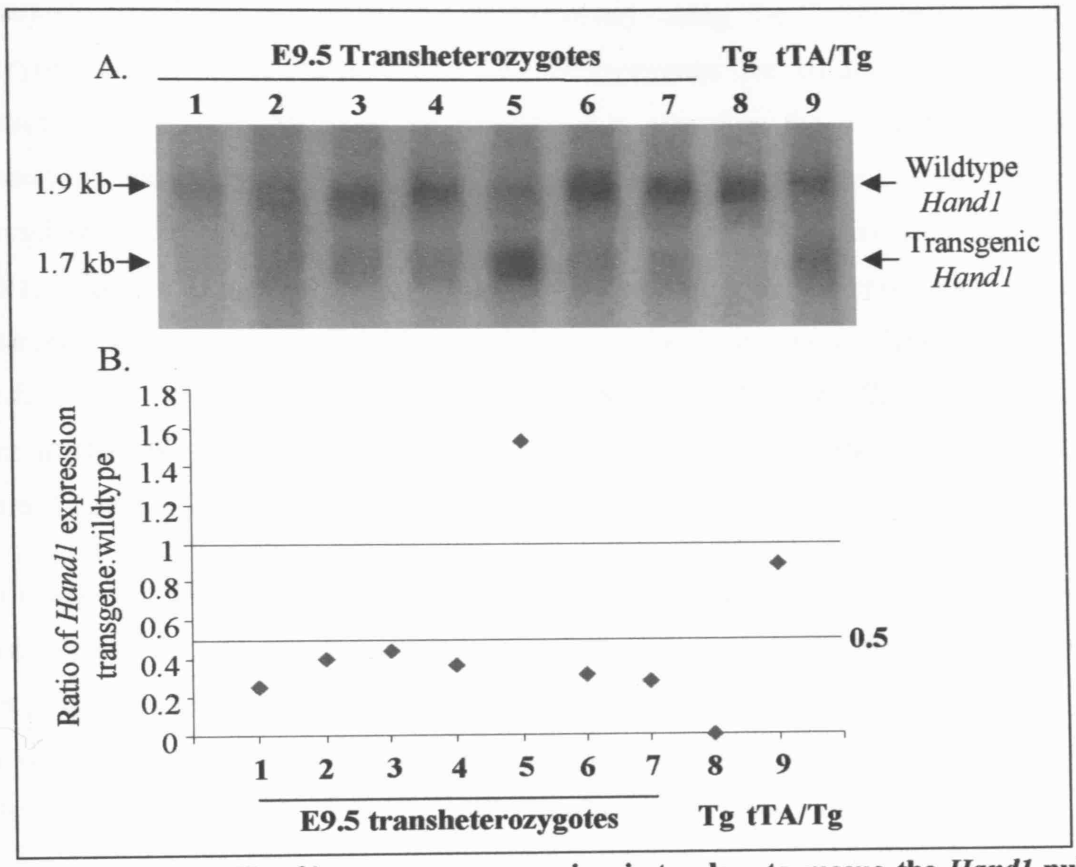


Figure 4.7. *Tre2-Hand1* transgene expression is too low to rescue the *Hand1*-null phenotype. (A.) E12.5 embryos were generated by crossing *Tet-Off-Hand1* heterozygote and *Tre2-Hand1* transgenic mice, poly (A) RNA extracted and northern blotting performed using a *Hand1* probe (see section 3.3.2.1.1). Lanes 1-7 are transheterozygote embryos, and accordingly express both the wildtype Hand1 message (1.9 kb) and the transgene message (1.7 kb). A *Tre2-Hand1* embryo that expresses only wildtype *Hand1* (lane 8) and an embryo known to express the *Tre2-Hand1* (lane 9) were included as controls. It is obvious in lanes 1-7 that the *Tre2-Hand1* transgene is not expressed at levels as high as the heterozygous level of wildtype *Hand1* within each individual sample. There is only one exception where the transgene is expressed at a higher level (lane 5). These observations were quantified by densitometry analysis using the Scion image program (B). This analysis confirmed that in the majority of embryos, the *Tre2-Hand1* transgene was expressed at less than half the level of heterozygous *Hand1*.

kb for *Tre2-Hand1*. All transheterozygote embryos expressed the transgene (Figure 4.7A). The levels of *Tre2-Hand1* transgene expression relative to wildtype *Hand1* expression were then estimated by densitometry using the Scion image program. Embryos that have the transactivator are heterozygous for wildtype *Hand1* as the transactivator is knocked-in to the *Hand1* locus, therefore the comparison made is between a heterozygous level of *Hand1* expression and the level *Tre2-Hand1* transgene expression. In only one embryo is the transgene expressed at a higher level than that of wildtype *Hand1* (Figure 4.7, lane 5). In the remaining six embryos, the levels of transgene expression do not even equal half of the heterozygous level of wildtype *Hand1*. This northern analysis also demonstrated that the *Tre2-Hand1* transgene is silent in the absence of a Tet-Off transactivator, as shown by the *Tre2-Hand1* only control (Figure 4.7, lane 8).

Unfortunately, the above expression analyses confirmed that the two *Tre2-Hand1* transgenic lines could not be used in conjunction with the *Tet-Off-Hand1* transactivator strain to generate the tet-inducible knock-out of *Hand1*. Fortunately, we have received as a kind gift a transgenic responder strain, *Tet-Hand1*, from a collaborating group who study *Hand1* over-expression in neonatal mice headed by Tim Mohun at NIMR.

4.3.3 *Tet-Hand1*

The *Tet-Hand1* strain was previously tested for appropriate transgene expression when crossed with a Tet-On transactivator line at NIMR. With this transactivator, levels of *Tet-Hand1* transgene expression were shown to be 3-4 fold higher than wildtype *Hand1* in neonatal mice (Figure 4.8; Ross Breckenridge, unpublished observations). Although the transgene was known to express, it was not known whether it would be expressed at similar levels when crossed with the *Tet-Off-Hand1* strain. As with the two *Tre2-Hand1* transgenic lines, it was attempted to assess the levels of *Tet-Hand1* relative to a heterozygous level of wildtype *Hand1* expression.

The *Tet-Hand1* strain was also crossed into the *Hand1*-null background, and subsequently into the *Tet-Off-Hand1* background, to attempt rescue of the *Hand1*-null phenotype by generating compound heterozygotes.

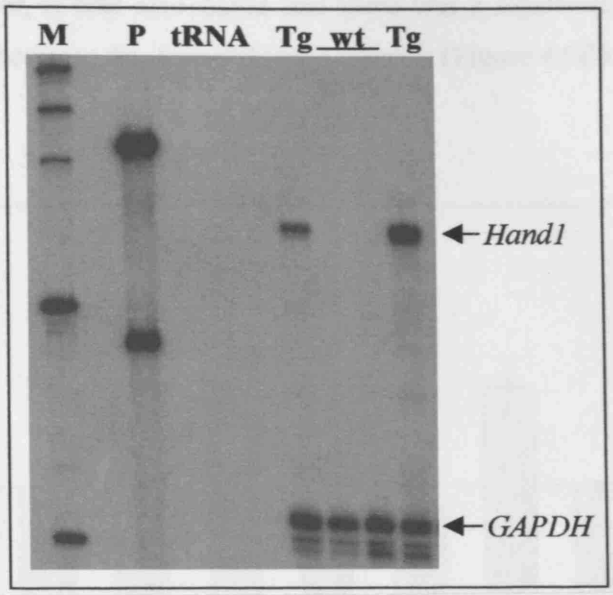


Figure 4.8. *Tet-Hand1* is expressed in neo-natal mice. (Figure provided by R. Breckenridge, unpublished observations) An RNase protection assay was performed to analyse the expression of *Tet-Hand1*, driven by a Tet-On transactivator, in neonatal mice (Tg) and wildtype controls (wt). tRNA was used as a negative control. M, marker; P, probes.

4.3.1 Tet-Hand1 expresses transgenic *Hand1* at a high level

In order to evaluate whether the *Tet-Hand1* strain expressed the *Hand1* transgene at high levels when induced by the *Tet-Off-Hand1* transactivator strain, two crosses were set up between *Tet-Off-Hand1* heterozygotes and *Tet-Hand1* transgenic mice. Embryos were dissected at E9.5, genotyped and poly(A) RNA was extracted from selected embryos. Northern blotting was performed using a *Hand1* probe on individual transheterozygote embryos, and controls were included that carried only the *Tet-Hand1* transgene. The northern blotting was unsuccessful, the probe appeared to be hybridising to the ribosomal RNA (not shown). This could reflect the low levels of poly(A) RNA used due to each sample representing a single E9.5 embryo. Since the northern blotting did not work, more crosses were set up between *Tet-Off-Hand1* heterozygotes and *Tet-Hand1* transgenic mice to generate more transheterozygote embryos. Embryos were again dissected at E9.5. Total RNA was isolated from individual embryos and used for real-time PCR analysis (Laurent Dupays, NIMR). Seven transheterozygote embryos, two transgene only controls and two wildtype controls were examined for relative levels of *Hand1* expression. Five out of the seven transheterozygote embryos tested exhibited elevated levels of *Hand1* expression, between 1.8 and 2.4 fold above wildtype (Figure 4.9A). *Hand1* expression levels in the remaining two embryos were comparable to

wildtype. However, it was also noted that there was a significant variation in *Hand1* expression levels between the four wildtype controls (Figure 4.9B).

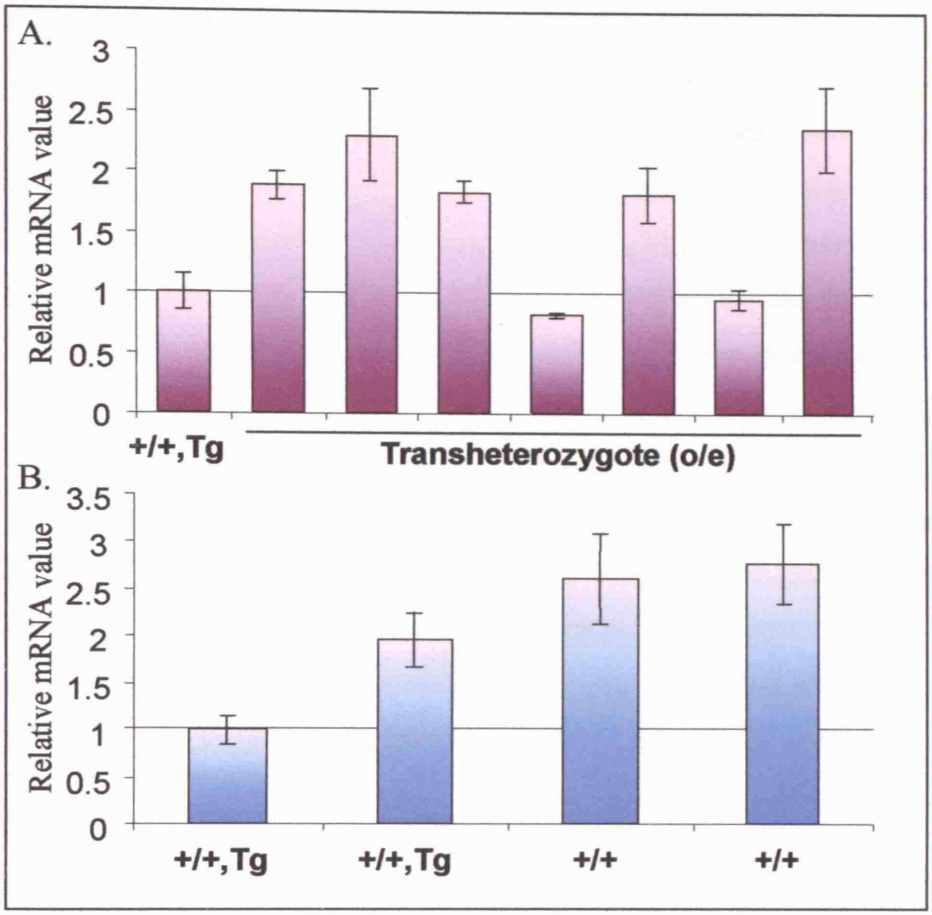


Figure 4.9. *Tet-Hand1* transheterozygote embryos express *Hand1* around two-fold higher than wildtype embryos. (A) Total RNA was extracted from whole *Tet-Hand1* transheterozygote (*Hand1*^{tTA}/*Hand1*^{tetO}) and control (transgene only; +/+,Tg) embryos at E9.5 and quantitative real-time PCR performed for *Hand1* transcripts (includes wildtype and transgenic *Hand1*). *Tet-Hand1* transheterozygote embryos express *Hand1* at around two-fold higher than wildtype in the majority of embryos tested. (B) Real-time PCR for *Hand1* expression was performed as above on wildtype (+/+) and transgene only (+/+,Tg) controls. Levels of *Hand1* expression were found to be highly variable between embryos, attributed to differing amounts of starting material as embryos were at slightly different stages of development.

4.3.3.2 Rescuing the *Hand1*-null phenotype with *Tet-Hand1* compound heterozygotes

The *Tet-Hand1* responder line is known to express the *Hand1* transgene at high levels in neonatal mice (R. Breckenridge, unpublished observations). It was hoped, therefore, that the *Tet-Hand1* transgene would also be expressed during embryonic development when induced by the *Tet-Off-Hand1* transactivator strain. Furthermore, it was hoped that the levels of expression from this transgene would be adequate to rescue the *Hand1*-null background in compound heterozygotes. The *Tet-Hand1* transgenic line was therefore crossed into the *Hand1* heterozygote background, and then crossed with the *Tet-Off-Hand1* strain with the intention of generating compound heterozygotes and rescuing the *Hand1*-null background. One litter of embryos have been dissected at E9.5, one at E12.5, and genotyped for compound heterozygotes, as before, a one in eight possibility (Figure 4.1). One of the four E9.5 embryos was a compound heterozygote but unfortunately exhibited the classical *Hand1*-null phenotype (not shown). Not one of the five E12.5 embryos genotyped as having all three necessary components. Additionally, live born litters have been tail tipped and genotyped. As yet, there have been no compound heterozygotes identified in 31 animals born.

However, since the numbers required to exclude rescue are high, repeat crosses are ongoing with the hope that rescue of the *Hand1*-null phenotype may still be achieved using the *Tet-Off-Hand1* and *Tet-Hand1* strains.

4.4 Discussion

The *Tet-Off-Hand1* strain was shown, by RT-PCR, to express the Tet-Off transactivator at timepoints at which *Hand1* is expressed. Additionally, the transactivator displays a similar expression pattern to *Hand1* in E9.5 embryos as shown by *in situ* hybridisation. However, the transactivator expression pattern was shown to exhibit a degree of mosaicism. The transactivator was also shown to be transcriptionally active, to be able to recognise and bind the tet response element *in vivo*, since when the *Tet-Off-Hand1* line was crossed with the *Tre2-Hand1*(CN12) and *Tet-Hand1* responder lines, the tet-responsive *Hand1* transgene was expressed in both cases.

Antibiotic resistance cassettes contain strong promoters and prokaryotic DNA and have been known to affect the expression of the targeted gene. Often, the presence of such a

cassette causes hypomorphic phenotypes (Nagy et al., 1998), with low levels of expression from the targeted region. Furthermore Shin et al., (1999) found that removal of the neo^r cassette from their tet responder strain resulted in leaky expression, whereas in the presence of the neo^r the responder strain was silent in the absence of transavctivation, indicating that the neo^r cassette was preventing expression of their responder strain.

The mosaicism in *Tet-Off-Hand1* embryos, and indeed fairly low level of transactivator expression in most embryos examined, is potentially caused by the presence of the PGKneo^r cassette, particularly since it is orientated in the same direction as the Tet-Off transactivator. The PGKneo^r cassette was intentionally flanked by *lox*P sites to enable its removal should the need arise. The PGKneo^r cassette could therefore be removed by crossing the transactivator strain with another strain that expresses Cre recombinase ubiquitously. It would be interesting to investigate whether removal of the PGKneo^r improved the expression pattern of the transactivator, making it more reliable and a more useful tool for inducing responder-derived *Hand1* expression.

It has been reported that there is a progressive reduction in Tet-Off transactivator mRNA through consecutive generations of tTACMV-M1 and -M2 transgenic mice suggesting that the long-term stability of the tet-system *in vivo* is poor (Fedorov et al., 2001). One explanation provided for the observed decrease was de novo DNA methylation, a phenomenon that has been identified upon introduction of foreign DNA into the mammalian genome in cell lines (Hertz et al., 1999) and in mice (Guy et al., 1997; Schumacher et al., 2000). Methylation may, therefore, be affecting the expression of the transactivator in *Tet-Off-Hand1* mice, contributing to the variation in expression pattern. On the other hand, if methylation is not the cause, the alternative mode by which the transactivator mRNA is being reduced in the tTACMV-M1 and -M2 strains may also be having an effect in the *Tet-Off-Hand1* strain.

It was also attempted to show Tet-Off transactivator expression and function in developing *Tet-Off-Hand1* embryos using a tet-responsive LacZ indicator strain, *Tet-LacZ*, which was generated by H. Bujard. The *Tet-Hand1* strain was crossed with the *Tet-Off-Hand1* strain, and embryos were dissected and stained for β-galactosidase (β-gal) activity. Unfortunately, this failed repeatedly and no evidence of blue β-gal staining was observed, not even the levels of background that have been reported for this strain (T. Mohun, personal communication). However, the *Tet-LacZ* line inherently

has variations between sublines and indeed some sub-lines have failed to express LacZ even when tested with multiple transactivator strains (T. Mohun, NIMR, personal communication), and this has caused the *Tet-LacZ* indicator strain to be far less useful than it promised to be. The mosaic expression of the transactivator could also have contributed to the failure with the *Tet-LacZ* line, but it is quite likely that it was the combination of *Tet-LacZ* sublines not expressing correctly and the mosaicism of *Tet-Off-Hand1* that hindered this particular analysis.

The *in vivo* kinetics of transactivator expression/repression in response to dox still remains to be determined specifically for the *Hand1* system. The *Tet-Off-Hand1* and *Tre2-Hand1* strains will be crossed. Dox will be administered to pregnant mothers (followed by maintenance on dox chow) and embryos will be collected over a time course following the first administration of dox. RNA will be extracted from transheterozygote embryos (*Hand1*^{tTA}; *Hand1*^{tetO}) and wildype with transgene (*Hand1*^{tetO}) littermate controls. Northern blot analysis will then be performed to determine the expression of the *Tre2-Hand1* transgene in the presence and absence of dox, specifically the length of time taken to completely repress *Tre2-Hand1* expression. This analysis would be completed if and when rescue of the *Hand1*-null phenotype is attained using this tet-inducible system.

Two independent lines of the transgenic *Tre2-Hand1* mice were tested for the ablility to express transgenic *Hand1*. The two lines differed according to integration site (random) and transgene copy number (copy number 3, *Tre2-Hand1*(CN3) and copy number 12, *Tre2-Hand1*(CN12)). The *Tre2-Hand1*(CN12) line expressed the transgene when induced by the *Tet-Off-Hand1* transactivator, but the *Tre2-Hand1*(CN3) line did not, as demonstrated by RT-PCR and northern blotting.

In the *Tre2-Hand1*(CN3) line, the transgene may have been inserted into a transcriptionally silent locus, preventing its expression even in the presence of the Tet-Off transactivator. Alternatively, the *Tre2-Hand1* transgene may be truncated at the 3' end, within the Tre2 region. This region is not easily checked due to its repetitive nature, and is not contained within the regions that were checked by PCR and Southern blotting. Tre2 consists of seven concatemerised tetracycline response elements (TRE)(see section 1.4.2) and if not all are present, it is likely that the amount of expression from the transgene is much reduced, or completely absent.

Tre2-Handl(CN12) can be induced to express transgenic *Handl* indicating that the responder functions appropriately *in vivo*. The variation seen in expression levels of transgenic *Handl* most likely reflects the variable levels of transactivator expression. Furthermore, *Tre2-Handl*(CN12) is silent (not 'leaky') in the absence of a transactivator, and is induced in the presence of a transactivator. *Tre2-Handl*(CN12) was therefore crossed into the *Handl* heterozygous background, and subsequently crossed with the *Tet-Off-Handl* strain in an attempt to generate compound heterozygotes, and rescue the *Handl*-null phenotype with the tet-inducible *Handl* system. Rescue would be expected to occur at a frequency of one in eight (Figure 4.1). Rescue was never achieved using the *Tre2-Handl*(CN12). Only one E8.5 embryo was identified as being a compound heterozygote, but this embryo displayed the classical *Handl*-null phenotype, suggesting that the levels of *Handl* expression from the tet-system were lower than the level of *Handl* required for normal development. As predicted from the preliminary embryo analysis, northern blotting and densitometry indicated that the levels of *Tre2-Handl* expression were insufficient. In the majority of embryos tested, the transgene was expressed at a level less than half that arising from embryos genotyping as conventional heterozygotes for *Handl*. If the amount of expression from the transgene is half the level from a single copy of wildtype *Handl*, it is very unlikely to be able to rescue the *Handl*-null phenotype. Together with the low level of transgene expression, the one in eight expected frequency and moreover, the mosaic expression pattern of the transactivator, the possibility of obtaining a rescue event with the combination of lines currently available (*Tre2-Handl*(CN12)*/Tet-Off-Handl*) is highly improbable. To this end, two further responder lines have been generated and will be tested and crossed with the *Tet-Off-Handl* strain as described in this chapter. Moreover, once the *Tet-Off-Handl* strain has been crossed with a Cre strain to remove the neo^r cassette further attempts can be made at generating the tet-inducible knock-out of *Handl* with all *Tre2-Handl* strains available.

It was already known that the *Tet-Handl* transgene was not inserted into a silent locus and that a transgenic *Xenopus* myosin light chain (XMLC) driven Tet-On transactivator could induce expression of *Tet-Handl* in postnatal mice (Ross Breckenridge, NIMR, unpublished observations). The XMLC promoter region used to direct the expression of the Tet-On transactivator has been demonstrated to direct expression of a LacZ transgene throughout the embryonic and adult heart in mice (Latinkic et al., 2004).

Here, it was demonstrated by real-time PCR on individual embryos, that it was expressed at a relatively high level, around two-fold above wildtype, during embryonic development when induced by the *Tet-Off-Hand1* transactivator, albeit with a degree of variation.

The real-time PCR results showed variable levels of *Hand1* expression even within the wildtype controls. The embryos used for this analysis were litter-mates, but they had not been somite matched. The variation in real-time results was thought to stem from differing amounts of starting material as some embryos were more developmentally advanced and thus it would be expected that they express *Hand1* at a higher level than a younger embryo. It would be useful to be able to use real-time PCR to assess transgene levels specifically against the wildtype levels within each embryo rather than relying on inter-embryo comparisons. This would require designing primers to specifically amplify the *Tet-Hand1* transgene message and not the wildtype *Hand1* message as is achieved with the primers for *Tre2-Hand1* message (see section 3.2.10).

The level of expression from this *Tet-Hand1* responder line was significantly higher than that from the *Tre2-Hand1*(CN12) responder line, which meant a greater chance of obtaining rescue of the *Hand1*-null phenotype with the tet-inducible *Hand1* system.

In order to attempt rescue of the *Hand1*-null phenotype, the *Tet-Hand1* responder was crossed into the *Hand1* heterozygous background, and subsequently crossed with the *Tet-Off-Hand1* strain. Embryos were dissected at two stages of development and genotyped. Live born litters were also produced and genotyped. As yet, there has only been one compound heterozygote generated, at E9.5, which presented with the classical *Hand1* phenotype. However, given that the numbers generated so far are very small (four embryos at E9.5 and five at E12.5) the possibility of obtaining rescue should not be ruled out. It may also be worthwhile to cross *Tet-Off-Hand1/Tet-Hand1* transheterozygotes to increase the probability of obtaining the correct genotype, and increasing levels of expression of both transactivator and transgene. For the reasons discussed above, and also given the relatively high levels of expression of transgenic *Tet-Hand1*, efforts to attain rescue are ongoing. However, it is predicted that rescue will most likely be a rare event given the putative mosaicism of the transactivator, and the one in eight chance of generating the correct genotype.

Nevertheless, a bimodal system has been created where, in addition to attempting to generate an inducible loss of function model for Hand1 on a null background, it is also possible to study gain of function for Hand1 on a wildtype background (see Chapter 5). The *Tre2-Hand1*(CN12) and *Tet-Hand1* transgenic responder strains were, therefore, crossed with the *Tet-Off-Hand1* strain to generate transheterozygote embryos on a wildtype background that potentially over-express *Hand1*, exclusively in *Hand1* expressing cells, thus enabling investigation of the effects of up-regulation of Hand1 in the developing heart at specific, key developmental stages.

Chapter 5: Employing the Tet-Off system to generate a gain of function model for Hand1

5.1 Introduction

As described previously (see Chapter 4) an advantage of utilising a transgenic responder strain is that it provides the opportunity not only to carry out loss of function studies, but also to carry out gain of function studies to decipher the role that Hand1 plays in the developing mouse heart. Transheterozygote embryos can be generated that carry both the Tet-Off transactivator and the transgenic responder on a heterozygous *Hand1* background (as the transactivator is targeted to the *Hand1* locus, see Figure 2.1). If expression levels from the *Tre2-Hand1* transgene are sufficiently high to exceed that from a single wildtype copy of *Hand1*, transheterozygote embryos will over-express *Hand1* exclusively in cells in which *Hand1* is normally expressed, since the transactivator is under control of the *Hand1* locus. As such, this represents an entirely novel gain of function approach, and enables investigation of *Hand1* over-expression in multiple lineages, including the developing heart and placenta.

5.1.1 *Hand1* over-expression studies to date

The majority of over-expression studies performed for *Hand1* have been designed to decipher the role of Hand1 in trophoblast differentiation (see section 1.2.10). It has been demonstrated that Hand1 is necessary and sufficient to promote terminal differentiation of trophoblast giant cells (Cross et al., 1995; Hemberger et al., 2004). It has also been shown that ectopic/over-expression of *Hand1* in the limb bud can induce ectopic digits (McFadden et al., 2002; Fernandez-Teran et al., 2003; Firulli et al., 2003). However, only one over-expression study has been performed in the developing mouse heart. Togi et al. (2004) targeted *Hand1* to the *MLC2v* locus by homologous recombination, and implicated Hand1 in interventricular septum formation and ventricular expansion (see sections 1.2.6.8-9).

Whilst the study performed by Togi et al. (2004) was informative in evaluating the effects of *Hand1* over-expression in the left and right ventricles, it also resulted in ectopic *Hand1* expression. Specifically, *Hand1* was mis-expressed throughout the entire

ventricular region, rather than being predominantly left-sided and restricted to the outer curvature of the left ventricle and only weakly expressed in the right ventricle. It is, therefore, of interest to examine the effects of *Hand1* over-expression restricted exclusively to *Hand1* expressing cells.

5.1.2 The tet-inducible Hand1 gain of function model

The gain of function model requires a transactivator strain expressing the Tet-Off transactivator specifically in *Hand1*-expressing cells, and a transgenic tet-responsive *Hand1* responder strain.

The *Tet-Off-Hand1* transactivator strain has been successfully targeted to the endogenous *Hand1* locus. The *Tet-Off-Hand1* strain has been shown to express the transactivator in accordance with *Hand1* expression, in the correct cell types and at the correct stages of development, and to function appropriately by transactivating a tet-inducible transgene (see Chapter 4). This strain was therefore used to generate the gain-of-function model.

Two transgenic responder lines, *Tre2-Hand1*(CN12) and *Tet-Hand1* (a kind gift from T. Mohun, NIMR), were available for the analysis of *Hand1* over-expression that have been shown to express transgenic *Hand1*. Induction of *Hand1* expression from the transgene in the *Tre2-Hand1*(CN12) responder line was shown to be less than half the level of heterozygous *Hand1* expression (see section 4.3.2.3). Consequently, it is unlikely that over-expression studies will be successful with this particular responder line. However, the *Tet-Hand1* responder line displays much greater levels of transgene expression (see section 4.3.3.1), and is sufficient to induce over-expression of *Hand1* in transheterozygote embryos. The cardiac *Hand1*-over-expression phenotype has been investigated in terms of morphology, alterations in gene expression and potential causative mechanisms.

5.2 Methods

5.2.1 Mouse maintenance and embryo generation

The *Tet-Off-Hand1* and the *Tet-Hand1* strains were used for the gain of function analysis, colonies were maintained as described in section 4.2.1. Transheterozygote

embryos were generated by crossing the two strains above, as described in section 4.2.2. Whole mount bright field photographs were taken as described in section 4.2.8.

5.2.2 Dox administration

Dox administration was adapted from Schonig et al., 2002. Mated females were injected intraperitoneally with 2 mg dox in 0.83 % saline (in 100 µl aliquots) on the day of the vaginal plug (E0.5). Females were starved overnight for 15 hours and then maintained constantly on dox chow (3 mg/g) until embryos were taken at E9.5. Further injections of the same were carried out at various stages of development. Three different regimes were attempted, two injections at E0.5 and E2.5 only, or five injections at E0.5, E2.5, E4.5, E6.5 and E8.5 and finally three injections at E0.5, E4.5 and E8.5.

5.2.3 Outflow tract measurements

The length of the outflow tract in the over-expression embryos and in wildtype controls was measured from photographs using morphometric measurements in magnification-matched photographs (Adobe® Photoshop®).

5.2.4 Tissue processing

Embryos and placentae were dissected out in PBS and fixed overnight in fresh 4% formaldehyde/PBS at room temperature. Embryos and placentae were embedded in wax and sectioned as described previously in section 4.2.9.

5.2.5 Haematoxylin and eosin staining of embryo sections

Slides were de-waxed in 100% histoclear (Raymond Lamb Diagnostics) twice for five minutes then re-hydrated through 100% ethanol twice, then 95%, 80% and 70% once each.

The slides were then placed in Meyer's haematoxylin solution for 5-10 minutes (depending on embryonic stage) and washed in dH_2O for one minute. The slides were then stained in 0.5 % eosin (aqueous) for one minute and again washed in dH_2O. The slides were air-dried, usually overnight, and then mounted with coverslips using DPX mountant.

5.2.6 *In situ* hybridisation on embryonic tissue sections

In situ hybridisation on embryonic tissue sections was carried out as described by Moorman et al.,(2001).

Embryos were embedded in wax and sectioned as described in section 4.2.9. The sections were de-waxed with two, seven minute incubations in histoclear followed by two minutes in 1:1 histoclear:ethanol. After de-waxing, sections were transferred to 100% ethanol, twice for two minutes. Then the sections were rehydrated through one minute in each 95%, 90%, 70% and 50% ethanol. The sections were washed twice for five minutes in DEPC-PBS, and then incubated in ProteinaseK working solution (20 μg/ml)(see Appendix 1) for eight minutes at 37°C. Next the sections were placed in 0.2% glycine/DEPC-PBS for five minutes, and washed twice more for five minutes in DEPC-PBS before post-fixing in 4% formaldehyde/DEPC-PBS with 0.2% glutaraldehyde for 20 minutes. The sections were then washed twice for five minutes each in DEPC-PBS. The slides were dried as much as possible by suction and the sections encircled using a wax pen (ImmEdge). The sections were incubated in prehybridisation buffer (see Appendix 1), in a 'humid box' (paper towels soaked in 50% formamide/2x SSC), for at least an hour at 70°C. The prehybridisation mix was replaced with hybridisation mix (see Appendix 1) containing the appropriate probe at an approximate concentration of 400 ng/ml, and incubated overnight at 70°C. The sections were then rinsed in 2x SSC (pH 7) and then washed twice for 15 minutes in 50% formamide/2x SSC (pH 7) at 65°C and three times for five minutes in PBST (see Appendix 1) at room temperature. Next the slides were blocked for at least 30 minutes in B-Block (see Appendix 1), then incubated overnight in anti-DIGoxigenin-AP Fab fragments (Roche) at 1:2000 in B-Block, at 4°C. The sections were then washed three times for five minutes in PBST and twice for five minutes in NTMT (see Appendix 1). The sections were developed overnight with NBT/BCIP (Roche) in NTMT in the dark at room temperature. The slides were then rinsed and eosin stained (see section 5.2.5), left to dry and mounted with cover slips using DPX mountant.

5.2.7 Immunofluorescence on embryo sections

Immunofluoresence was performed on embryo sections with antibodies against cleaved caspase 3 (Cell Signalling Technology) and phospho-histone H3 (Upstate). For antibody details see Appendix 4.

Embryos were embedded in wax and sectioned as described in section 4.2.9, the sections were then de-waxed and rehydrated as described above in section 5.2.6 and circled with an ImmEdge pen. The sections were permeabilised with 0.5% Triton X-100/PBS for five minutes and blocked in 1% BSA in PBS (block) for 30 minutes. The slides were incubated with the primary antibody at the appropriate dilution (see Appendix 4) in block in a humidity chamber overnight at 4°C. A secondary antibody only coverslip was included as a control, and was left overnight in block. The cells were washed in block three times, for five minutes each. The slides were then incubated with the filtered secondary antibody, again at the appropriate concentration diluted in block (see Appendix 1), in a humidity chamber at room temperature for one to three hours. The slides were washed as above. Nuclei were stained as described in section 3.2.15. Cells were mounted with coverslips secured in 50% glycerol/PBS and fluorescence was visualised using a Zeiss Akioskop2 microscope and photographed using a FujiFilm FinePix S2 Pro digital camera. Cells were stored in the dark at 4°C.

5.2.8 Whole mount embryo *in situ* hybridisation

In situ hybridisation was performed on whole embryos as described in section 4.2.8 using riboprobes specific to *ANF*, *Hand1*, *MLC2v*, *Nkx2.5* and *Wnt11*. See Appendix 5 for probe details.

5.2.9 Optical projection tomography

Optical projection tomography (OPT) was carried out as described by (Sharpe et al., 2002). OPT was performed in collaboration with Tim Mohun's group at NIMR, using a prototype OPT scanner provided by Edinburgh Human Genetics Unit (HGU). The data analysis was performed using the OPT software, again provided by Edinburgh HGU, along with the Mouse Atlas software for manipulating wlz volumes. The volume rendering was done using Improvision Volocity 3.1.

5.2.10 Quantitative real-time PCR

Quantitative real-time PCR was performed by Laurent Dupays, NIMR. Total RNA was extracted from whole embryos or isolated hearts using Trizol (see section 3.2.6.1). First strand cDNA was synthesised using SuperScript II RT (see section 3.2.9.2). Quantitative real-time PCR was performed as described in section 4.2.14 for *ANF*,

Chisel, *Cx40*, *Cx43*, *GATA-4*, *Hand2*, *Irx4*, *Mef2c*, *Nkx2.5 and SRF*. Primer sequences are listed in Appendix 3.

5.3 Results

Two transgenic responder strains were available that are known to express transgenic *Hand1*, *Tre2-Hand1*(CN12) and *Tet-Hand1*. Therefore, in attempts to generate the gain of function, *Hand1* over-expression model, both of these strains were crossed with the *Tet-Off-Hand1* strain. As a result of these crosses, transheterozygote embryos were generated that carry the Tet-Off transactivator and the tet-responsive *Hand1* transgene (*Hand1*^{tTA};*Hand1*^{tetO}; Figure 4.1), that depending on levels of transgene expression, have the potential to over-express *Hand1* exclusively in *Hand1* expressing cells. Transheterozygote embryos from both transgenic responder strains were examined for a resultant phenotype.

5.3.1 *Tre2-Hand1*(CN12) transheterozygote embryos are normal

The *Tre2-Hand1*(CN12) line expresses transgenic *Hand1* at a level less than half that arising from a single wildtype copy of *Hand1* (see section 4.3.2.3). Therefore, it was not expected that transheterozygote embryos would over-express *Hand1*. Nevertheless, crosses were set up between *Tet-Off-Hand1* and *Tre2-Hand1*(CN12) mice, embryos were dissected at E8.5 and E12.5 and yolk sacs were taken for genotyping. Embryos were examined at E8.5 since *Hand1* expression is known to be crucial at this stage and it coincides with the onset of cardiac looping. Embryos were also examined at E12.5. *Hand1* expression is highest in the developing heart at around E10.5 and any abnormalities should, therefore, be evident by E12.5.

Transheterozygote embryos were generated at levels greater than expected according to Mendelian ratios, with seven E8.5 and 26 E12.5 embryos being produced during the crosses described above (Table 5.1); none of which ever displayed an abnormal phenotype. The relatively high number of transheterozygote embryos produced is most likely a result of having only generated a low number of embryos in total. A single E10.5 litter was also generated with one transheterozygote identified that displayed a normal phenotype. Additionally, expected ratios of transheterozygotes were produced from the live born litters (Table 5.1), again without apparent phenotype. As with the loss of function studies, this is most likely due to low levels of transgenic *Hand1*

expression (see section 4.3.2.3). Therefore, a responder line is required that can be induced to express *Hand1* at greater levels in order to generate transheterozygote embryos that over-express *Hand1* accordingly.

Table 5.1. ***Proportion of transheterozygotes generated for Tre2-Hand1(CN12)***

	$Hand1^{tTA}/Hand1^{tetO}$ (overexpression)	Total	Expected frequency
E8.5	7	30	4
E12.5	26	43	11
Post-natal	17	107	15

5.3.2 *Tet-Hand1* transheterozygote embryos over-express *Hand1*

In order to evaluate whether a gain of function model could be generated with the *Tet-Hand1* strain obtained from T. Mohun, NIMR, the *Tet-Off-Hand1* and the *Tet-Hand1* strains were crossed as above to generate transheterozygote embryos ($Hand1^{tTA}$; $Hand1^{tetO}$). E9.5 transheterozygote embryos were observed for any indication that *Hand1* was over-expressed in these embryos, and for any resultant phenotype. E9.5 was chosen as a time point as *Hand1* expression is high in the developing heart at this stage and over-expression may, therefore, affect the morphogenesis of the heart tube.

An abnormal phenotype was observed in E9.5 transheterozygote embryos that affects the regions of the heart in which *Hand1* is expressed, namely the outflow tract and the presumptive left ventricle (Figure 5.1) coincident with Tet-Off transactivator expression (see section 4.3.1.2). The phenotype is also in keeping with previous observations that Hand1 plays a cell autonomous role in cardiac morphogenesis. The outflow tract appeared to be elongated, protruding further away from the body wall both ventrally and laterally, and the presumptive left ventricle appeared to be thick-walled. Other embryological lineages appeared morphologically normal without any expansion, except the placentae, which appears to be developing abnormally in one moderately affected over-expression placenta examined (see section 5.3.13). The morphological aspects of the over-expression phenotype were examined by light microscopy, histological staining of embryonic sections and optical projection tomography (OPT). OPT can be used to capture images of intact embryos from many different angles and employs computed tomography to calculate a high-resolution 3D reconstruction using the inherent autofluorescence in embryos (Sharpe et al., 2002). OPT was, therefore,

Figure 5.2. The *Hand1* over-expression phenotype varies in severity. Rendered optical projection tomography of embryos at E9.5 from left, ventral and right views, of a wildtype containing *Tet-Hand1* transgene (+/+ Tg) control embryo (A-C), a moderately affected *Hand1* over-expression (o/e) embryo in which the outflow tract is slightly extended and the ventricle is reduced in size but otherwise appears normal (D-F) and a severely affected *Hand1* over-expression embryo that has a greatly extended outflow tract, abnormal looping and a small left ventricle (G-I). Red lines mark the extension of the outflow tract (C, F, I), and white arrows indicate the presumptive left ventricle (B, E, H). The extension of the outflow tract causes displacement of the heart ventrally, away from the body wall, and results in a gap forming between the outflow tract and the atria (white lines A, D, G). Scale bars approximately 100 μM

Movies showing virtual sections through the rendered OPT of the E9.5 wildtype containing *Tet-Hand1* transgene control embryo, the moderately affected *Hand1* over-expression embryo and the severely affected *Hand1* over-expression embryo are contained within Appendix 6 (CD), which can be found inside the back cover.

employed in order to explore the morphology of the *Hand1*-over-expression hearts further than was possible with the more conventional methods.

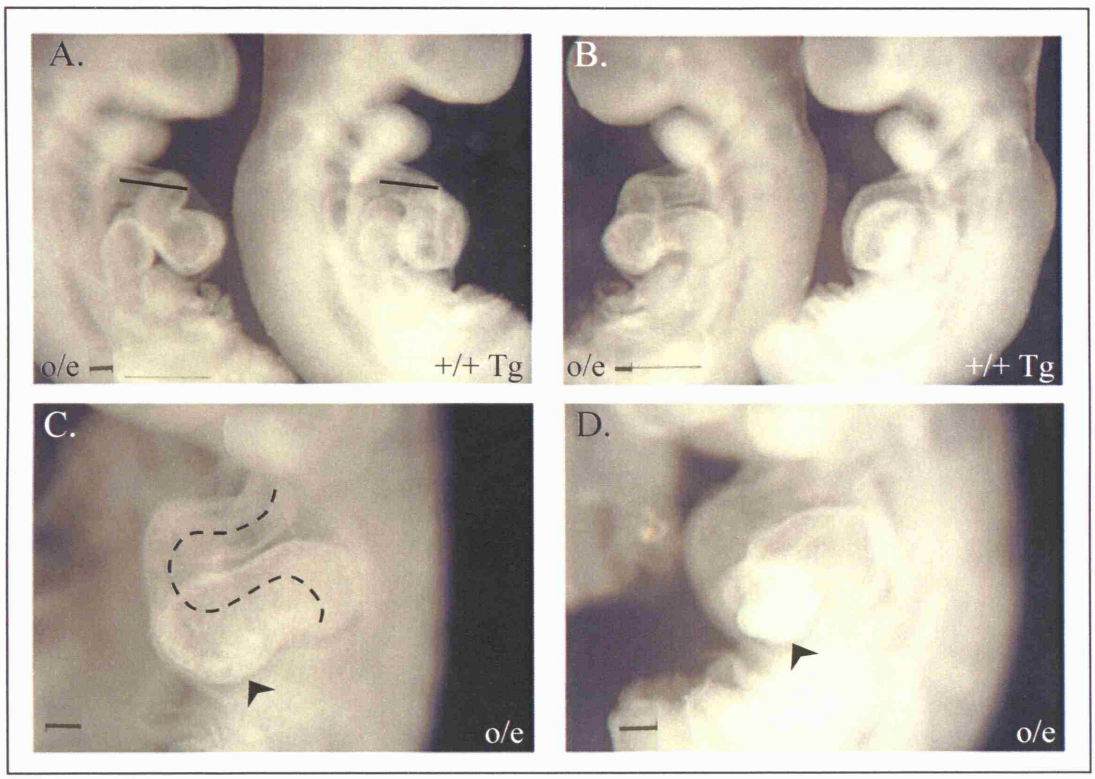
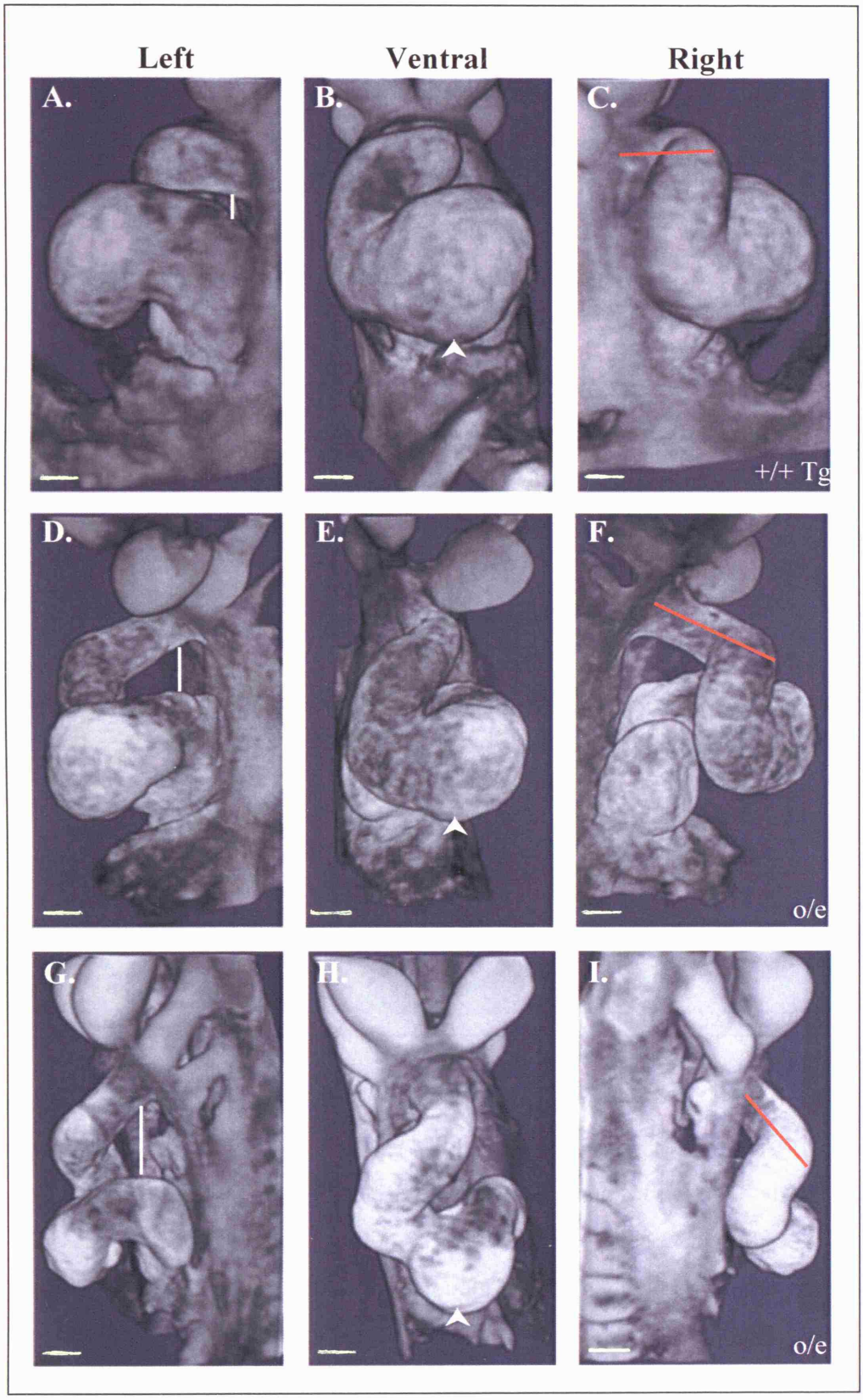


Figure 5.1. The *Hand1* over-expression phenotype. *Hand1* over-expression at E9.5 induces extension of the outflow tract, abnormal looping and defective left ventricular development. (A) Right lateral view (B) Left lateral view of bright-field whole mount *Hand1* over-expression (o/e) and wildtype with *Tet-Hand1* transgene (+/+ Tg) control embryos, black lines indicate extended outflow tract. (C, D) Left lateral view of *Hand1* over-expression embryos highlighting the abnormal looping (dashed line in C) and small, unexpanded left ventricle (arrows in C and D). Scale bars approximately 100 μM

5.3.3 Three classes of *Hand1* over-expression phenotype

There is variation in the severity of the over-expression phenotype in transheterozygote embryos most likely attributable to the variation observed in transactivator expression pattern and levels in the *Tet-Off-Hand1* strain (see section 4.3.1.3). This has resulted in a range of hypermorphic phenotypes. There are two broad classes of over-expression phenotype, moderately affected and severely affected (Figure 5.2, D-F and G-I respectively). In the most severely affected cases, which comprise 17% of the total number of transheterozygote embryos, the embryos present with an extended outflow tract, and the presumptive ventricle is small and necrotic, and does not appear to have


Left
Ventral
Right
A.
B.
C.
+/+ Tg
D.
E.
F.
o/e
G.
H.
I.
o/e

ballooned out from the primary heart tube (Figure 5.2G-I). Additionally, these severely affected embryos tend to be relatively fragile. The moderately affected embryos (56%) have an extended outflow tract and reduced ventricular size but the ventricle is not necrotic and has undergone a degree of expansion (Figure 5.2D-F). A third class, that represents a much smaller fraction of transheterozygote embryos (5%), appeared to be defective only in the presumptive left ventricle, similar to that observed in the severely affected embryos. The ventricle is greatly reduced in size and appears to have with thickened walls with no chamber ballooning, but the outflow tract is of normal length (Figure 5.3). Additionally, some transheterozygotes appear to be unaffected (21%)(not shown).

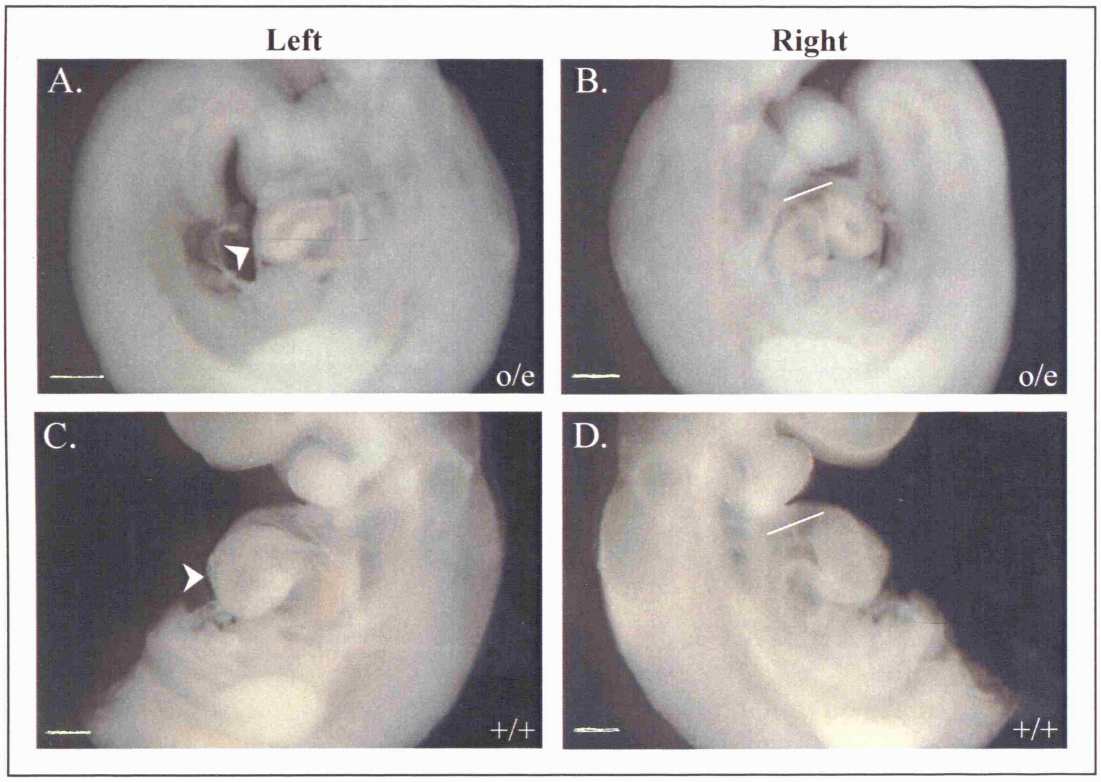


Figure 5.3. The left ventricle only is affected in a small sub-set of *Hand1* over-expression embryos. Left (A,C) and right (B,D) lateral views of bright-field whole mount embryos, *Hand1* over-expressing with ventricle only affected phenotype (o/e; A,B) and wildtype (+/+; C,D) at E9.5. The ventricle is small, thick-walled and has not ballooned out from the primary heart tube in the *Hand1* over-expressing embryo (A) compared to the larger, ballooned wildtype ventricle (C)(white arrows), but the outflow tract is of normal length (B) and is comparable to wildtype (D) (white lines).

Scale bars approximately 200 μM

5.3.4 The outflow tract is elongated in *Hand1* over-expression embryos

From initial observations of whole E9.5 over-expression embryos, it was clear that the outflow tract was elongated to varying degrees (Figure 5.4A-C). In order to confirm this observation, outflow tract length was measured in over-expression embryos (n=20) and compared to transgene positive wildtype (n=9) controls (Figure 5.4D). In severely affected embryos, the outflow tract was found to be around twice as long as the wildtype controls. In the moderately affected embryos, outflow tracts were found to be approximately 50% longer than wildtype controls. In the over-expression embryos in which only a ventricular defect had been noted, the outflow tract length was found to be equivalent to wildtype (not shown).

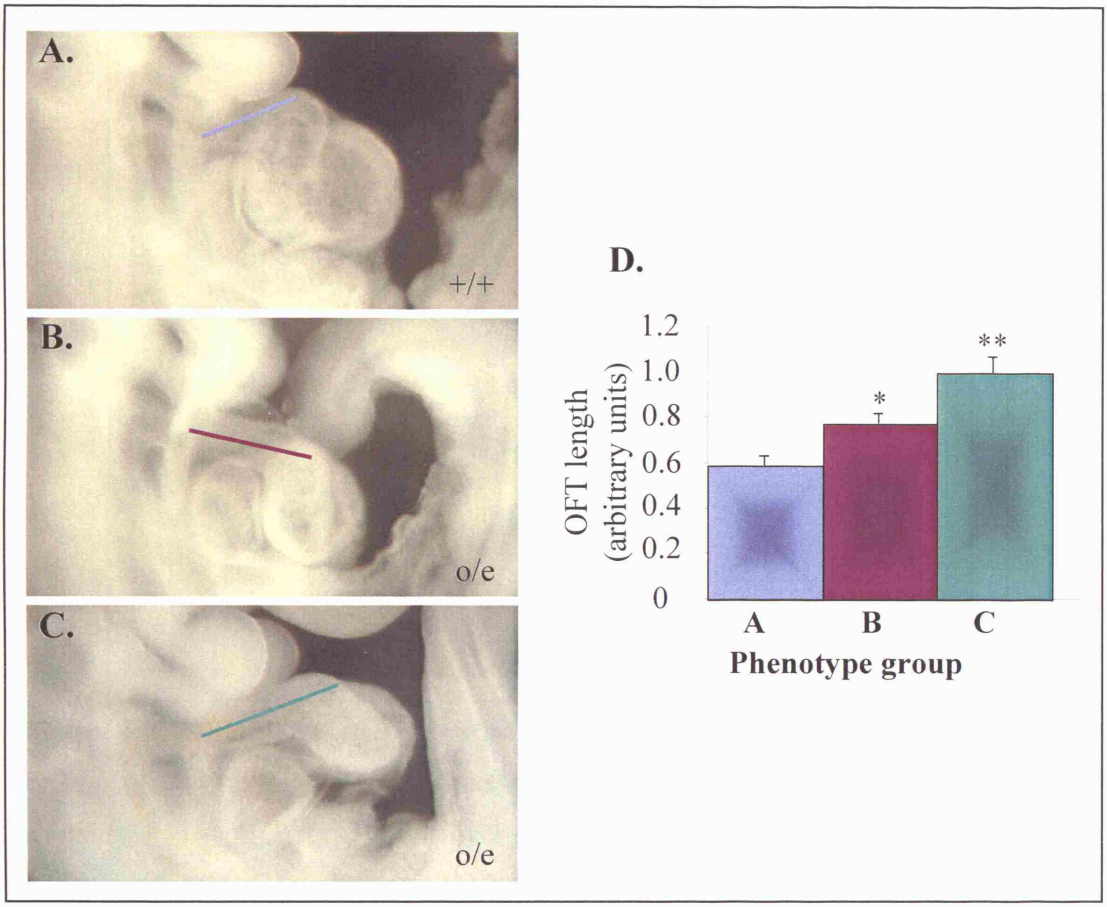


Figure 5.4. The outflow tract is elongated in *Hand1* over-expression embryos. (A-C) Bright-field whole mount right lateral view of wildtype (+/+)(A) and moderately affected (B) and severely affected (C) *Hand1* over-expressing (o/e) embryos at E9.5. Coloured lines indicate outflow tract extension. (D) Outflow tract mean length measurements, quantified in relative units based on length of coloured lines in A-C, from n=9 wildtype control (A), n=11 moderately affected (B) and n=5 severely affected embryos (C). * $p<0.05$, ** $p<0.01$.

Transheterozygote over-expression embryos were also embedded in wax, sectioned and then stained with haematoxylin and eosin. This analysis further emphasised the abnormal morphology of the over-expression embryos. In frontal sections, the cross-section of the outflow tract can be seen to be distinctly circular rather than elongated as seen in the wildtype embryo, indicating that the outflow tract has extended further away from the body wall in the over-expression embryo before looping (Figure 5.5A and B).

In addition to highlighting the outflow tract extension, the OPT images of *Hand1*-over-expression embryos demonstrated that the extension of the outflow tract was accompanied by a gap between the atria and the outflow tract that is not normally seen in wildtype embryos, presumably since the extended heart tube was displaced ventrally, away from the body wall (Figure 5.2, C wildtype, F and I over-expression).

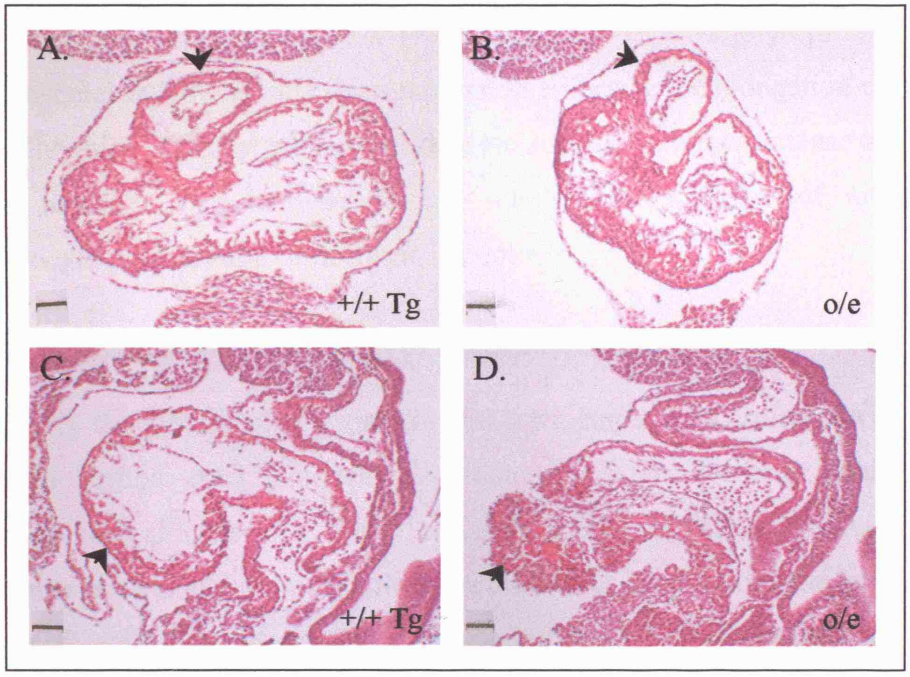


Figure 5.5. Histological sections confirm the *Hand1* over-expression phenotype. Frontal (A,B) and sagittal (C,D) sections through control wildtype with *Tet-Hand1* alone (+/+ Tg)(A,C) or *Hand1* over-expressing (o/e) moderately affected (B) or severely affected (D) embryos at E9.5. Sections were stained with haematoxylin and eosin. In the frontal sections, note the reduced ventricular size and the distinctly circular cross section of the outflow tract (B) in the over-expressing embryo compared to the elongated wildtype outflow tract and normal size ventricle (A). The sagittal sections confirm the ventricle is small, densely packed and lacks a lumen (D).

Scale bars approximately 50 μM

5.3.5 The presumptive left ventricle is thick-walled and lacks a defined lumen

In the most severe cases, *Hand1* over-expression in the presumptive left ventricle results in a small, necrotic ventricle as evident in both whole mount photographs (Figure 5.1) and OPT analysis (Figure 5.2G-I), and a reduced ventricular size in the moderately affected embryos (Figure 5.2D-F). In sagittal sections through over-expression embryos, those most severely affected revealed that the presumptive ventricle consisted of a mass of densely packed cells, some of which appeared hypertrophic, with no lumen and no trabeculae developing, and has not begun to balloon out from the primary heart tube (Figure 5.5D). In moderately affected over-expression embryos, frontal sections confirmed the reduced ventricular size, and that the ventricle appeared to be developing normally otherwise, with trabeculae forming and a lumen present (Figure 5.5B).

In the transheterozygote *Hand1* over-expression embryos, the elongation of the outflow tract and the thick, densely packed ventricle are suggestive of an increase in cell number in these regions. An increase in cell number is indicative of either elevated cardiomyocyte proliferation or decreased apoptosis.

5.3.6 Apoptosis is normal in over-expression embryos

Apoptosis is thought to play a part in the later stages of cardiac development, for instance in outflow tract remodelling, although this has not been confirmed in the mammalian heart (Fisher et al., 2000), and also in valve formation (Keyes and Sanders, 2002), but is not thought to be significant in the earlier stages when the heart tube is still growing and expanding. Nevertheless, it was important to rule out the possibility that a lack of apoptosis was causing the phenotype observed in the E9.5 over-expression embryos. Immunofluorescence on sagittal embryo sections using an antibody to cleaved caspase 3 (Cell Signalling Technology) demonstrated that levels of apoptosis in the transheterozygote over-expression embryos was equivalent to the levels in wildtype embryos (Figure 5.6), and additionally indicated that apoptosis was a rare occurrence at this stage of normal cardiac morphogenesis.

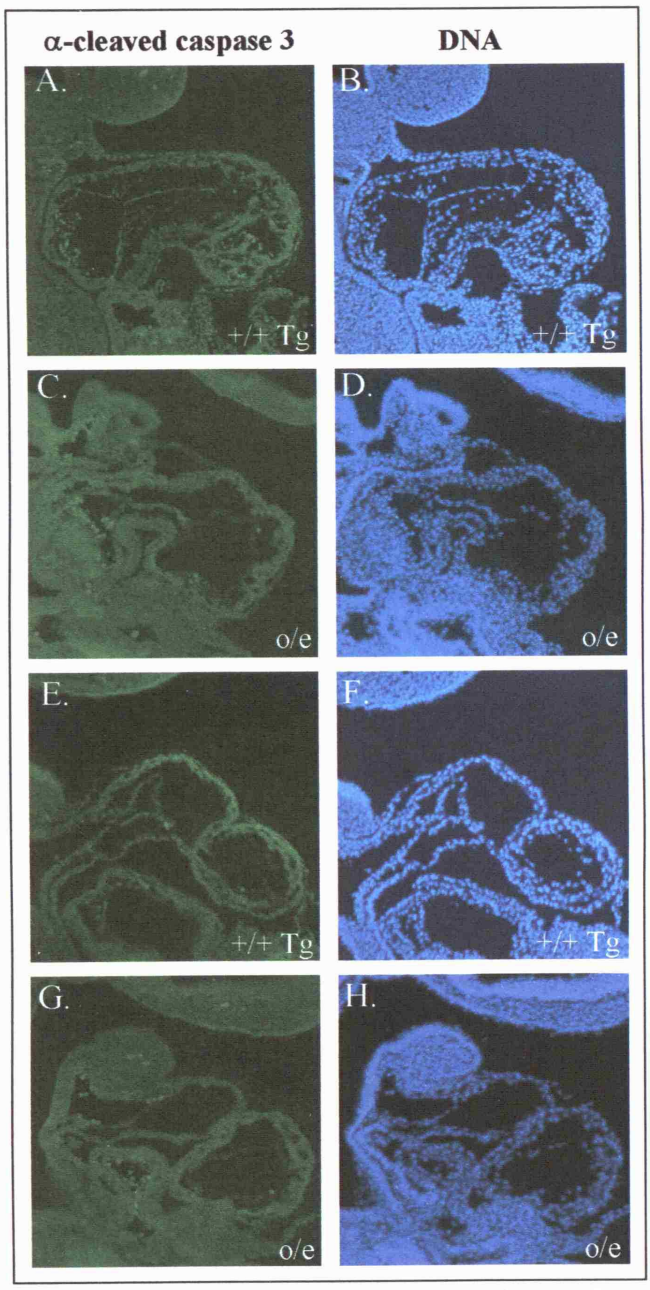


Figure 5.6. Apoptosis is normal in *Hand1* over-expression embryos. Frontal (A-D) and sagittal (E-H) sections through control (wildtype with *Tet-Hand1* alone (+/+ Tg); A,B and E,F) or *Hand1* over-expressing (o/e);(C, D and G,H) embryos at E9.5. Sections were immunostained with an α-cleaved caspase 3 antibody (Cell signalling) to detect cells undergoing apoptosis followed by a fluorescein conjugated (FITC) secondary antibody (A, C, E, G) and counterstained with bis-benzamide to detect cell nuclei (B, D, F, H). The levels of apoptosis at this stage of development were low, and were equivalent between wildtype and *Hand1* over-expressing embryos.

5.3.7 Increased proliferation in the over-expression embryos

The developing mouse heart has been shown to have different rates of proliferation in different regions of the heart as early as the linear heart tube stage (Sedmera et al., 2003). One of the areas that is documented to normally display only low levels of proliferation is the outflow tract. The outflow tract increases in size by way of recruitment of cardiomyocytes from the secondary heart field (Kelly et al., 2001; Kelly and Buckingham, 2002), the myocytes in the outflow tract therefore have a low proliferative capacity.

Therefore, it was determined whether the outflow tract and developing ventricle were proliferating at a higher rate than normal in the over-expression embryos. Immunofluorescence was performed on sagittal and frontal embryo sections using an antibody to phospho-histone H3 (Upstate) to mark cells in metaphase, revealed there was an increase in cell proliferation in the walls and cells within the lumen of the distal outflow tract (Figure 5.7) in the over-expression embryos compared to wildtype embryos. As mentioned above, the outflow tract normally increases in length as myocardium is added from the anterior heart field (AHF)(see section 1.1.3.3). However, since *Hand1* is not expressed in the AHF it is unlikely that the increased number of proliferating cells in the outflow tract of *Hand1*-expressing embryos originates from the AHF, but that Hand1 is only affecting cells within the OFT. As observed in previous studies (Riley et al., 1998), these observations confirm that Hand1 plays a cell autonomous role in cardiac morphogenesis.

Interestingly, *in situ* hybridisation for *Nkx2.5* on frontal embryo sections revealed that the 'extra' cells within the lumen of the outflow tract of *Hand1* over-expression embryos are *Nkx2.5* negative (Figure 5.8). This suggests that the 'extra', proliferative cells are immature and defective in differentiation.

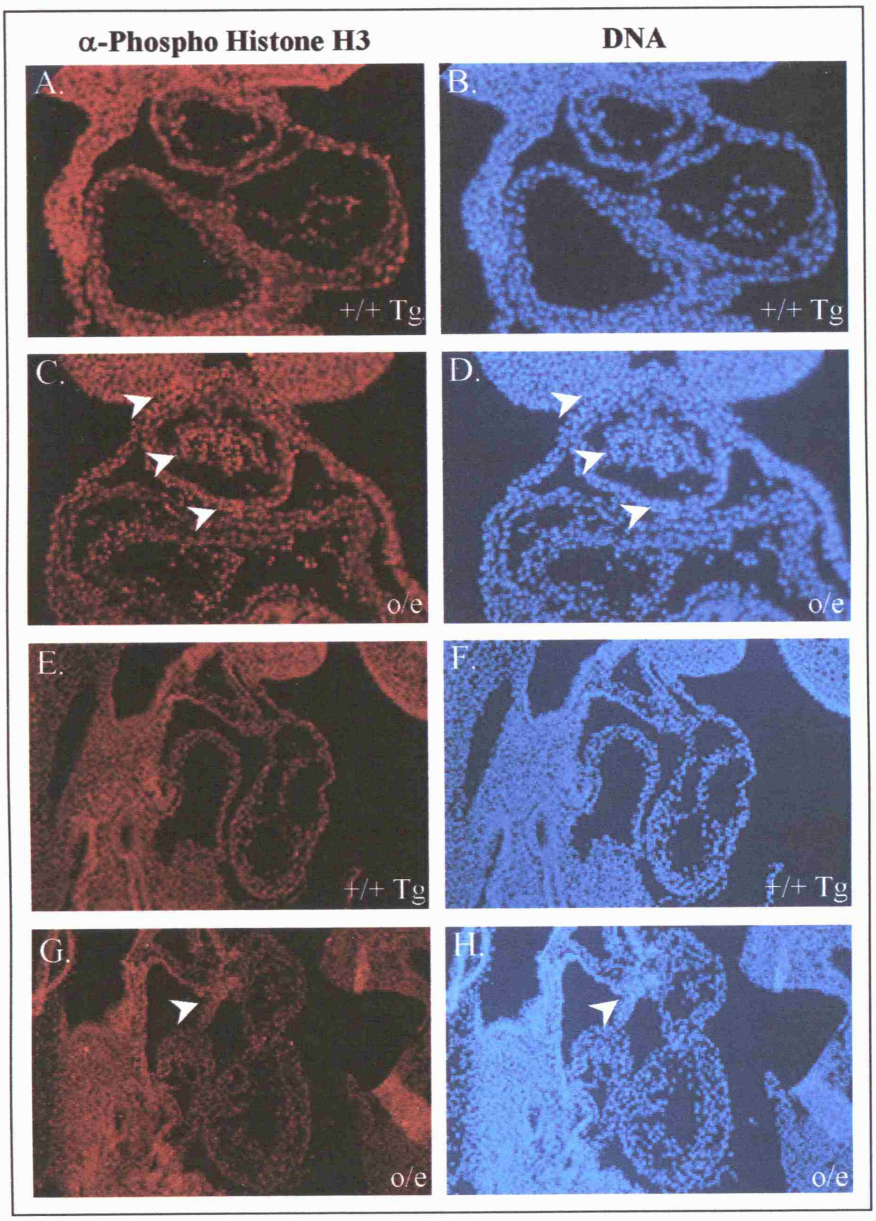


Figure 5.7. *Hand1* over-expression results in an increase in proliferation in the distal outflow tract. Frontal (A-D) and sagittal (E-H) sections through control (wildtype with *Tet-Hand1* alone (+/+ Tg); A,B and E,F) or *Hand1* over-expressing (o/e);(C, D and G,H) embryos at E9.5. Sections were immunostained with an α-phospho-histone H3 antibody (Upstate) to detect mitotically active cells followed by a rhodamine conjugated (TRITC) secondary antibody (A, C, E, G) and counterstained with bis-benzamide to detect cell nuclei (B, D, F, H). White arrowheads highlight a region of elevated proliferation of cells in the outflow tract of the *Hand1* over-expressing embryo compared to the control.

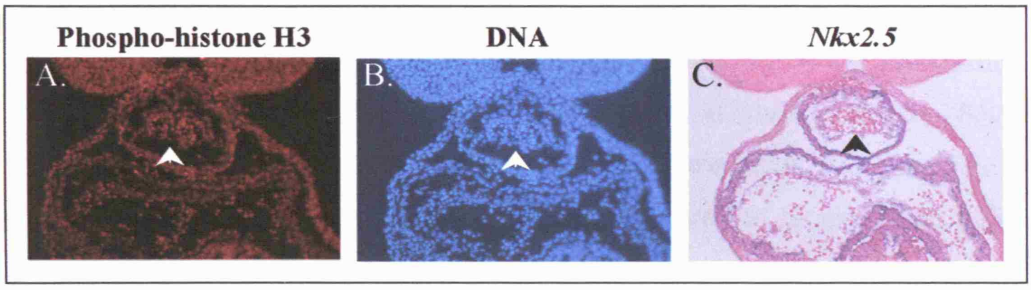


Figure 5.8. *Nkx2.5* is down-regulated in the 'ectopic' cells in the outflow tract of *Hand1* over-expression embryos. Frontal sections through *Hand1* over-expression embryos at E9.5. (A) Immunostained with α-phospho-histone H3 as described in Figure 5.7 and (B) counterstained with bis-benzamide. (C) RNA *in situ* hybridisation on sections for *Nkx2.5* transcripts. White arrows in (A) and (B) indicate the 'extra', proliferative pool of cells within the lumen of the outflow tract and the black arrow in (C) indicates that the extra cells are negative for *Nkx2.5* expression.

5.3.8 Markers of cardiomyocyte differentiation are down-regulated

Given that there is an increase in cardiomyocyte proliferation in the *Hand1*-over-expressing embryos, it was predicted that there may be a concomitant defect in cardiomyocyte differentiation. Furthermore, the fact that the ventricle in the most severe cases was small, but packed with cells that were not forming trabeculae or ballooning out from the primary heart tube, both of which are associated with differentiation, was suggestive of a defect in appropriate cardiomyocyte differentiation. Additionally, as mentioned above, the proliferating cells in the lumen of the outflow tract were *Nkx2.5* negative, a further indication that there may be a deficiency in differentiation of cardiomyocytes where *Hand1* is over-expressed. Markers of cardiomyocyte differentiation were therefore examined by both *in situ* hybridisation, on whole embryos and on embryo sections, and by real-time PCR on whole embryos and on isolated hearts. Various cardiac markers were identified as down-regulated in *Hand1* over-expressing embryos by both *in situ* hybridisation and real-time PCR, which suggests that over-expression of *Hand1* does result in defective cardiomyocyte differentiation.

Wnt11 was selected as marker of outflow tract differentiation at E9.5 (Cai et al., 2003). In addition to this, Wnt11 has been shown to induce cardiomyocyte differentiation in

Xenopus embryos and the P19 cell line (Pandur et al., 2002), and also in mouse ES-cell derived embryoid bodies (Terami et al., 2004). Given the expression pattern of *Wnt11* and its putative role in cardiomyocyte differentiation, *in situ* hybridisation was performed to *Wnt11* transcripts in *Hand1*-over-expression whole embryos. *Wnt11* was observed to be down-regulated in over-expression outflow tracts, being restricted to the outer edge whereas in wildtype embryos *Wnt11* appeared to be expressed throughout the outflow tract (Figure 5.9A-B). The embryos upon which *in situ* hybridisation had been performed were embedded in wax and sectioned to examine internal structures. This confirmed the down-regulation of *Wnt11* in the outflow tract (Figure 5.9C-D).

Mlc2v was used as a marker for differentiation of ventricular myocytes (O'Brien et al., 1993). *In situ* hybridisation was performed for *Mlc2v* on whole transheterozygote embryos and a reduction in *Mlc2v* expression was observed in the ventricle of over-expression embryos (Figure 5.10). In addition, expression of *Mlc2v* in the outflow tract also appeared to be greatly reduced (Figure 5.10). The embryos were sectioned as above, but the signal was too weak to be observed on individual sections (not shown).

ANF is a marker for differentiated secondary, or working, myocardium in the four chambers of the developing heart (Christoffels et al., 2000). Particularly, *ANF* marks trabeculated myocardium, which is one of the more differentiated myocardial populations. *ANF* expression in *Hand1*-over-expressing embryos was therefore analysed by *in situ* hybridisation on whole embryos in comparison to wildtype controls. Whole mount *in situ* hybridisation to *ANF* transcripts showed expression in wildtype embryos to be high throughout the trabeculated myocardium of the presumptive ventricle as expected, but also showed expression in the proximal outflow tract (Figure 5.11A). This restriction of *ANF* to the proximal outflow tract suggests that ordinarily, the proximal and distal portions of the outflow tract may have distinct differentiation profiles. In moderately affected *Hand1* over-expressing embryos, *ANF* is clearly down-regulated in the trabeculae of the ventricle (Figure 5.11E-F). This down-regulation is highlighted by subsequent sectioning of the embryos subjected to *in situ* hybridisation (Figure 5.11G-H). In addition, the boundary of *ANF* expression in the proximal outflow tract was reduced in *Hand1* over-expressing embryos (Figure 5.11A-D).

Figure 5.9. *Wnt11* expression is down-regulated in the outflow tract of *Hand1* over-expression embryos. (A-B) Bright field whole mount E9.5 wildtype with *Tet-Hand1* transgene (+/+ Tg; A) and moderately affected *Hand1* over-expression (o/e; B) embryos viewed from the right lateral aspect following *in situ* hybridisation to *Wnt11* transcripts. *Wnt11* expression marks the outflow tract at E9.5 and is seen to be down-regulated in the outflow tract of moderately affected *Hand1* over-expression embryos (white arrows). (C,D) Sections through E9.5 embryos following whole mount *in situ* hybridisation to *Wnt11* transcripts confirms down-regulation of *Wnt11* in the outflow tract of moderately affected *Hand1* over-expression embryos (arrows).

Figure 5.10. *Mlc2v* expression is down-regulated in *Hand1* over-expression embryos. Bright-field whole mount E9.5 wildtype with *Tet-Hand1* transgene (+/+ Tg; A) and moderately affected *Hand1* over-expression (o/e; B) embryos viewed from the left lateral aspect following *in situ* hybridisation to *Mlc2v* transcripts. *Mlc2v* expression marks the ventricles (white arrows) at E9.5 and is also expressed in the distal portion of the outflow tract (black arrows). *Mlc2v* is down-regulated in the ventricle of moderately affected *Hand1* over-expression embryos and expression in the outflow tract appears to be eliminated. Expression in the foregut endoderm (f), however, is unchanged in *Hand1* over-expression embryos compared to control.

Scale bars approximately 100 μM

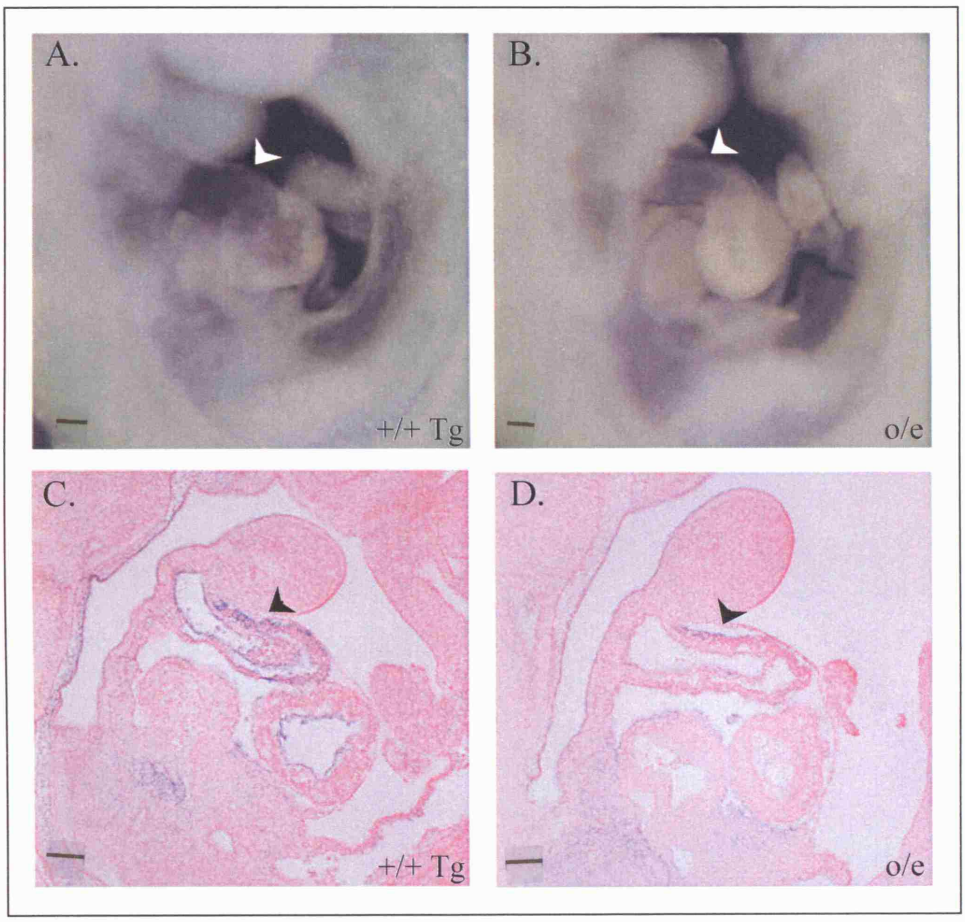


Figure 5.9.

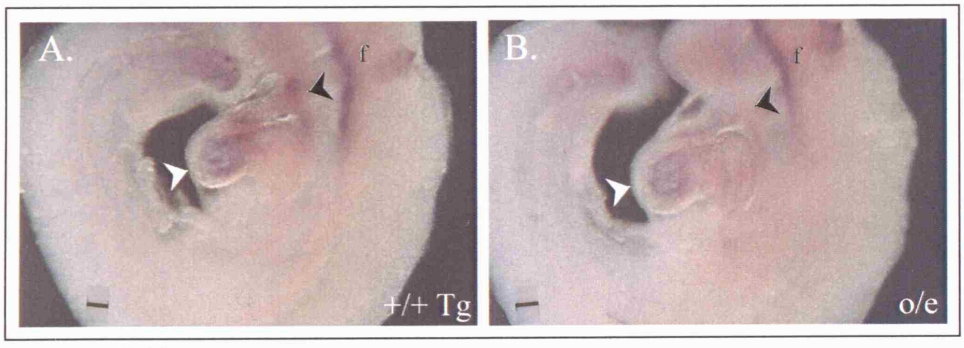
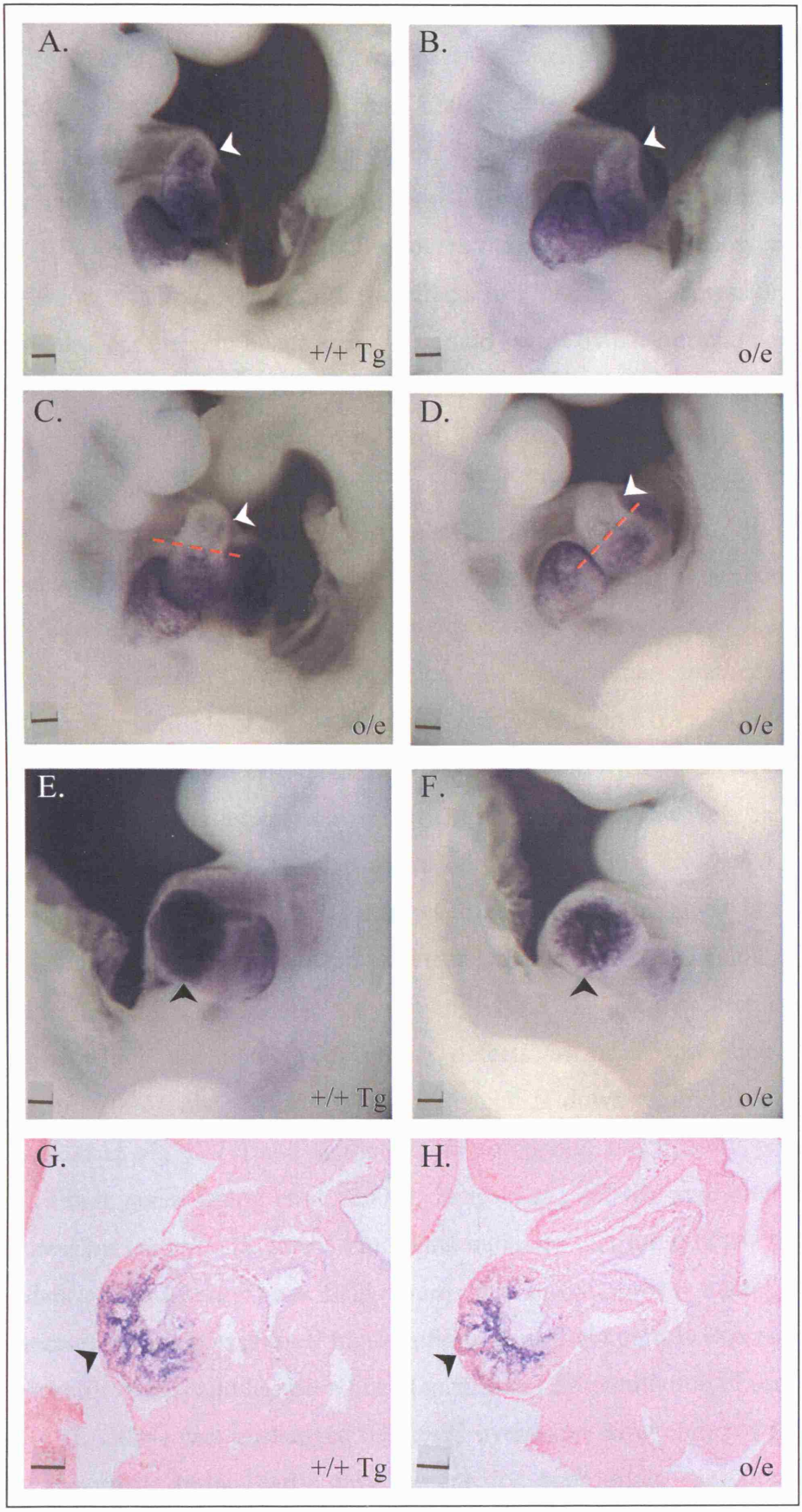


Figure 5.10.

Figure 5.11. *ANF* is down-regulated in *Hand1* over-expression embryos. (A-F) Bright field whole mount E9.5 wildtype with *Tet-Hand1* transgene (+/+ Tg; A, E) and moderately affected *Hand1* over-expression (o/e; B, C, D, F) embryos viewed from the right lateral aspect (A-D) and left lateral aspect (E,F) following *in situ* hybridisation to *ANF* transcripts. *ANF* expression marks the trabeculated chamber myocardium at E9.5. *ANF* expression can also be seen in the proximal outflow tract (A). *ANF* is down-regulated in the ventricle (black arrows; E,F) and outflow tract (white arrows; A-D) of moderately affected *Hand1* over-expression embryos. Red dashed line indicates altered boundary of *ANF* expression in the proximal outflow tract of moderately affected *Hand1* over-expression embryos. (G,H) Sections through E9.5 embryos following whole mount *in situ* hybridisation to *ANF* transcripts confirms down-regulation of *ANF* in the presumptive left ventricle of moderately affected *Hand1* over-expression embryos (arrows). Scale bars approximately 100 μM


A.
+/+ Tg
B.
o/e
C.
o/e
D.
o/e
E.
+/+ Tg
F.
o/e
G.
+/+ Tg
H.
o/e

5.3.9 Expression of other cardiac markers

Various other markers were analysed by real-time PCR using primers to amplify *ANF*, *Chisel*, *Cx40*, *Cx43*, *GATA-4*, *Hand2*, *Irx4*, *Mef2c*, *Nkx2.5* and *SRF*. Total RNA was isolated from whole embryos, seven *Hand1* moderately affected over-expression, two wildtype and two wildtype with the *Tet-Hand1* transgene only and quantitative real-time PCR was performed for the genes listed above. However, as described previously (see section 4.3.3.1), unless the embryo had a severe over-expression phenotype i.e. greatly elevated levels of *Hand1* expression, the effects of *Hand1* over-expression on cardiac gene regulation appeared to be masked to a certain extent by background noise from the remainder of the embryo that is not affected. It was also noted that between wildtype embryos there could be differing levels of gene expression, likely caused by the embryos being at slightly different stages of development (see Figure 4.9). Nevertheless, the analysis has been performed in order to provide an insight into any global and significant changes in cardiac marker gene expression. The reduced level of *ANF* in severely affected *Hand1* over-expressing embryos was confirmed by quantitative real-time PCR. Additionally, *Chisel*, another marker of working myocardium (Christoffels et al., 2000) along with *Nkx2.5* and *GATA4* which are early markers of cardiomyocyte differentiation, were also identified as significantly down-regulated in *Hand1* over-expression embryos (Figure 5.12). However, the ventricular-specific homeobox gene *Irx4* and markers of gap junctions, *Cx40* and *Cx43*, were unchanged (Figure 5.12). Mef2c is thought to be critically involved in regulation of AHF differentiation as a downstream target of Isl1 and GATA4 (Dodou et al., 2004), and Foxh1 (Von, I et al., 2004)(see section 1.1.3.3.2). Neither *Mef2c* expression, changes in which are thought to reflect defects in AHF formation (Kelly and Buckingham, 2002), nor *Hand2* expression, which is downstream of Mef2c in same pathway (Lin et al., 1997) and is thought to co-operate functionally with Mef2c to activate cardiac genes (Zang et al., 2004a; Zang et al., 2004b) were altered in *Hand1* over-expression embryos (Figure 5.12). This indicates that the outflow tract defect is independent of the anterior heart field contribution to the outflow tract. Additionally, *SRF*, which is a widely expressed transcription factor that controls expression of many cardiac specific genes, and has been linked to terminal differentiation of cardiomyocytes (Miano et al., 2004) was unchanged in *Hand1* over-expressing embryos (Figure 5.12). SRF is important during early development for appropriate mesoderm formation (Arsenian et al., 1998), and a cardiac specific knock-out of *SRF* has indicated that it lies

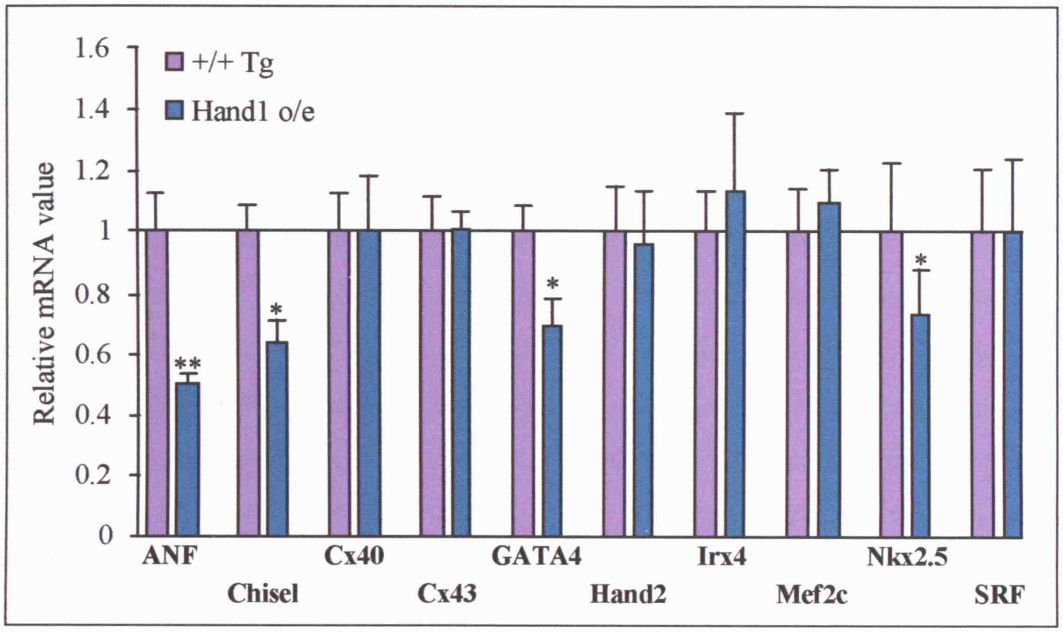


Figure 5.12. Expression of cardiac genes in *Hand1* over-expression embryos. Total RNA was extracted from wildtype with *Tet-Hand1* transgene control (+/+ Tg) and *Hand1* over-expression severely affected (o/e) whole E9.5 embryos and quantitative real-time PCR analysis was performed for a variety of cardiac differentiation markers. Levels of *Cx40*, *Cx43*, *Hand2*, *Irx4*, *Mef2c* and *SRF* were not altered in *Hand1* over-expression embryos. However, *ANF*, *Chisel*, *GATA4* and *Nkx2.5* were found to be significantly down-regulated in severely affected *Hand1* over-expressing embryos. * $p<0.05$; ** $p<0.01$.

upstream of key cardiac transcription factors including Nkx2.5, GATA4 and myocardin (Parlakian et al., 2004). Therefore, Hand1 presumably also lies downstream of SRF.

In an attempt to reduce the levels of background noise, hearts were dissected out from E9.5 embryos, total RNA was extracted, first strand cDNA synthesised and real-time PCR performed for the same panel of cardiac markers as described above. However, the results gained from this assessment were unreliable (not shown), which was attributed to inconsistent amounts of embryonic starting material.

5.3.10 *Hand1* over-expression phenotype is prevented by addition of doxycycline

In order to verify that the phenotype in transheterozygote embryos was caused by an over-expression of *Hand1* from the *Tet-Hand1* transgene, it was attempted to switch off transgene expression by the addition of doxycycline, rescuing the over-expression phenotype. Pregnant females were injected intraperitoneally with doxycycline two, three or five times throughout pregnancy, and additionally maintained throughout on dox chow. Embryos were dissected at E9.5 and examined for any manifestation of the over-expression phenotype.Two injections (at E0.5 and E2.5) were insufficient to prevent the over-expression phenotype, despite the pregnant females also being maintained constantly on dox chow. Yolk sac genotyping identified a single transheterozygote embryo that displayed a moderate level of over-expression phenotype, with an extended outflow tract and reduced ventricular size.

Injecting pregnant females five times (at E0.5, E2.5, E4.5, E6.5 and E8.5) appeared to have an adverse effect on the developing embryos. One litter was fully resorbed by E9.5. In a second litter, half of the embryos (four of eight) were dying regardless of genotype (Figure 5.13). However, the single transheterozygote embryo in this litter was unaffected and developing normally without the over-expression phenotype.

Figure 5.13. High doses of doxycycline adversely affect embryonic development. Five injections of dox given to pregnant females combined with maintenance on dox chow (3 mg/g) appeared to affect embryonic development to varying degrees, regardless of genotype. Half of the litter was dying as illustrated by these three examples. tTA, transactivator heterozygote; +/+, wildtype. Scale bars approximately 200 μM

The most successful regime was injecting doxycycline three times at E0.5, E4.5 and E8.5. All embryos appeared healthy and developed normally. Five transheterozygote embryos were produced, all of which were developing normally (Figure 5.14). The outflow tract of only one over-expression embryo was measured, and the length was equivalent to a wildype littermate, and fell in the range previously identified for wildtype outflow tract length (see section 5.3.4; Figure 5.4).

5.3.11 The heart tube is elongated at E8.0

To determine whether the linear heart tube was affected by *Hand1* over-expression at earlier stages, embryos were dissected at E8.0. Yolk sacs were taken for genotyping and transheterozygotes were compared to wildtype litter-mate controls. In keeping with the phenotype at E9.5, the heart tube at E8.0 appears to be longer in the *Hand1* over-expressing embryos (Figure 5.15). At this stage of development, *Hand1* is expressed in the anterior and posterior portions of the linear heart tube.

5.3.12 Unaffected over-expression embryos are morphologically normal at E12.5 and E14.5

Severely affected Hand1 over-expression embryos were fragile and necrotic and appeared to be dying, presumably from heart failure. However, moderately affected *Hand1* over-expressing embryos were not necrotic and were of normal size, suggesting that they may recover and survive. Therefore, to ascertain whether the over-expression of *Hand1* at E9.5 affected later cardiac morphogenesis and whether transheterozygote embryos were surviving, embryos were dissected at E12.5 and E14.5, embedded in wax and sectioned. Sections from E12.5 and E14.5 transheterozygote embryos were stained for haematoxylin and eosin and compared to wildtype litter-mates to uncover any potential morphologic defect. All transheterozygote embryos examined appeared to be developing normally at these stages, with a normal outflow tract and normal left ventricle (not shown). Furthermore, transheterozygotes occur at expected Mendelian frequencies at all time points analysed, including live born litters (see Table 5.2). As yet, no defect is apparent in transheterozygote mice that survive to post-natal stages.

Figure 5.14. Addition of doxycycline prevents the manifestation of the *Hand1* over-expression phenotype. Bright-field whole mount transheterozygote (tTA/Tg; A,B) and wildtype (+/+; C,D) E9.5 dox-treated embryos from left lateral view (A,C) and right lateral view (B,D). A total of six transheterozygote embryos have been observed so far that exhibit no apparent over-expression phenotype (A,B), when compared to wildtype control embryos (C,D). Scale bars approximately 200 μM

Figure 5.15. The primary heart tube is expanded in E8.0 *Hand1* over-expression embryos. Bright-field whole mount left lateral views of hearts from somite matched wildtype (+/+; A,C) and *Hand1* over-expression embryos (o/e; B,D). Embryos were dissected before turning (9 somites (s); A,B) and during turning (12 somites; C,D). The primary heart tube is expanded and looping appears accentuated (arrows) in *Hand1* over-expression embryos, and as looping progresses the heart tube becomes more expanded and displaced ventrally (indicated by white lines).
Scale bars approximately 50 μM

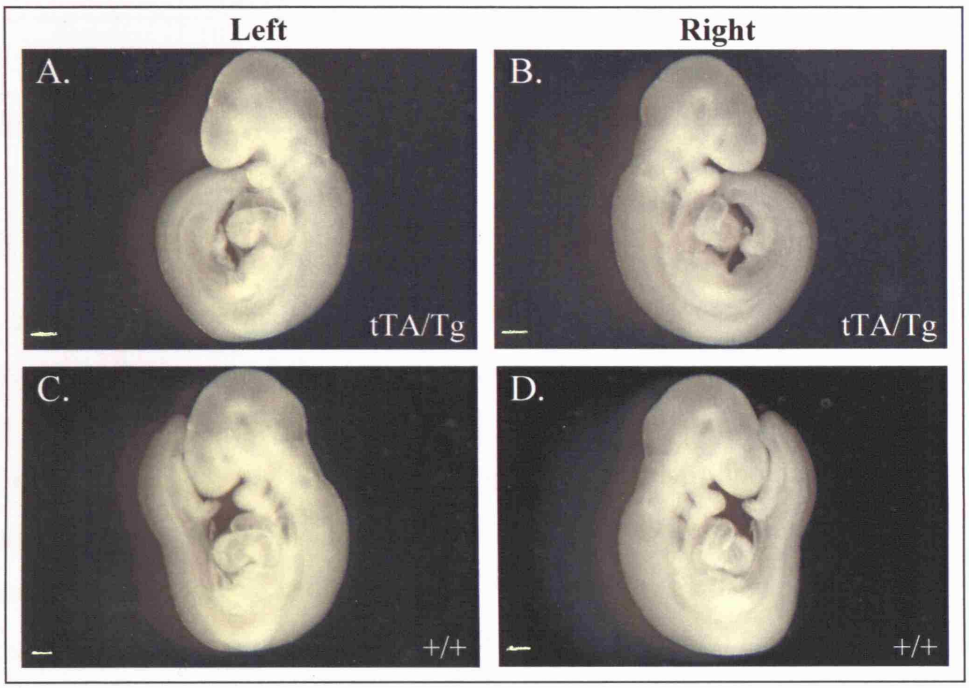


Figure 5.14.

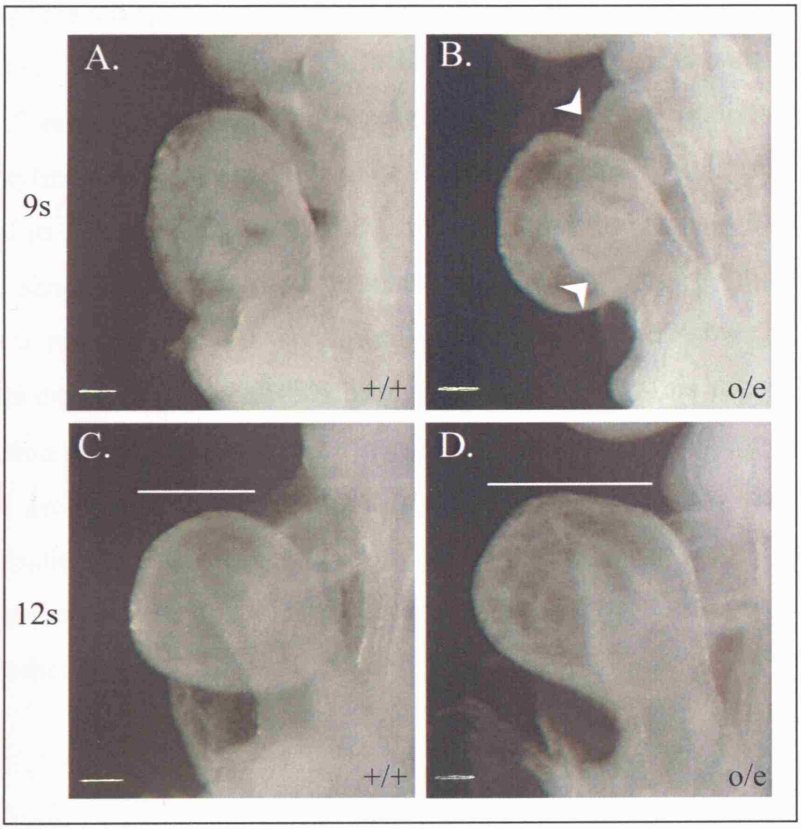


Figure 5.15.

Table 5.2. ***Proportion of transheterozygotes generated for Tet-Handl***

	***Handl*^{tTA}/*Handl*^{tetO} (over-expression)**	**Total**	**Expected frequency**
E9.5	103	385	96
E12.5	8	26	7
Post-natal	16	59	15

5.3.13 Placentae

Since the Tet-Off transactivator is targeted to the endogenous *Handl* locus, it is expressed in all regions of the developing embryo in which *Handl* is expressed (see section 4.3.1.2). It is, therefore, expected that in transheterozygote embryos, *Handl* would be over-expressed in the other lineages, such as the trophoblast of the developing placenta (see section 1.2.5.2). Furthermore, *Handl* over-expression is known to induce differentiation of trophoblast giant cells (see section 1.2.10). In addition, Handl inhibits Mash2, which is essential for maintaining the diploid spongiotrophoblast layer that underlies the trophoblast giant cell layer, by sequestration of E-factors such as ALF-1 (mouse E12 orthologue)(Scott et al., 2000). Therefore, it might be expected that over-expression of *Handl* may result in an increase in the number of trophoblast giant cells, or a hypoplastic spongiotrophoblast layer, or a combination of the both. Placentae were dissected from a moderately affected *Handl* over-expression embryo and a wildtype E9.5 embryo, embedded in wax and sectioned. The sections were examined by haematoxylin and eosin staining. The placenta appears hypoplastic in the *Handl* over-expression placenta (Figure 5.16), in that it seems thinner and does not form the typical cone shape. This could reflect elevated levels of Handl inhibition of Mash2 resulting in a reduction in the spongiotrophoblast layer. However, since only one placenta over-expressing *Handl* has been examined so far, this aspect of the *Handl* over-expression phenotype requires further, more detailed investigation. It is interesting to note that embryos over-expressing *Handl* survive despite the potential placental hypoplasia, indicating that the defect is not as severe as that of loss of *Handl* function where a reduction in the trophoblast layer, and particularly reduced giant cell number, is embryonic lethal (Riley et al., 1998).

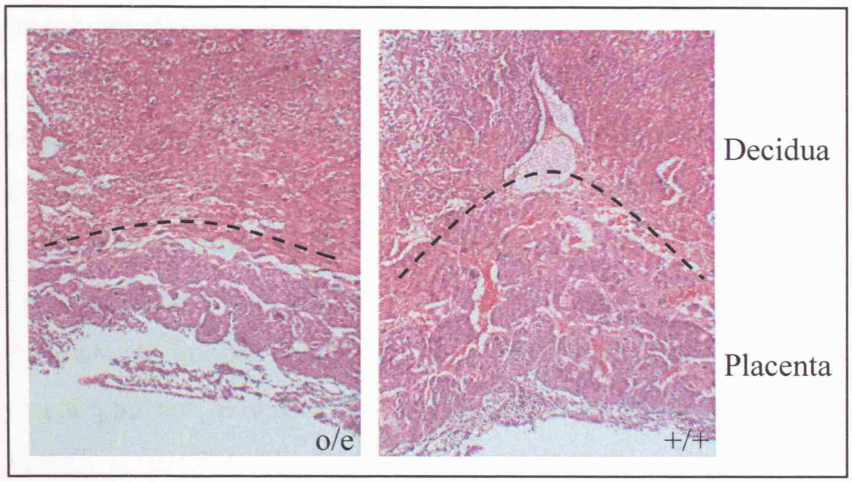


Figure 5.16. *Hand1* over-expression affects placental development. Sections through control wildtype (+/+) or moderately affected *Hand1* over-expressing (o/e) placentae from E9.5 embryos. Sections were stained with haematoxylin and eosin. Note the reduced placental thickness and the flatter curvature (dashed line) in the *Hand1* over-expressing placenta compared to the typically cone-shaped (dashed line), normal size wildtype placenta.

5.4 Discussion

One of the major aims of this project was to generate a gain of function model for Hand1, exclusively in *Hand1*-expressing cells, with which to study the effects of *Hand1* over-expression and underpin a precise role for Hand1 within the developing mouse heart. This gain of function approach was not successful with the *Tre2-Hand1*(CN12) responder line, which was due to low levels of transgenic *Hand1* expression (see section 4.3.2.3). However, a gain of function model was successfully generated in transheterozygote embryos from crosses between the *Tet-Off-Hand1* knock-in and *Tet-Hand1* transgenic strains. *Hand1* over-expressing embryos have an extension of the outflow tract and small thick-walled ventricles. It was demonstrated that the *Hand1* over-expression phenotype is a cell autonomous effect, caused by an increase in cell proliferation at the expense of cardiomyocyte differentiation, and that in the outflow tract, this was independent of the contribution from the anterior heart field.

Since the Tet-Off transactivator has been targeted to the endogenous *Hand1* locus (see Chapter 2), the transactivator is expressed solely in *Hand1* expressing cells. At E9.5, the transactivator is co-expressed with Hand1 in the outflow tract and presumptive left ventricle (see section 4.3.1.2; Figure 4.3). Accordingly, in the E9.5 *Hand1* over-

expressing transheterozygote embryos, the regions of the heart that are affected are the outflow tract, which is elongated and loops abnormally, and the presumptive left ventricle, which is small and densely packed with cells. Presumably, the Tet-Off transactivator is driving expression of the *Tet-Hand1* transgene in these regions and thus, causing over-expression of *Hand1*. In support of this, the administration of dox prevents the over-expression phenotype. In the presence of dox the Tet-Off transactivator is unable to bind the tet response element, therefore expression of the *Tet-Hand1* transgene can not be induced. Consequently, in dox-treated transheterozygote embryos, *Hand1* is presumably expressed at endogenous levels and development proceeds normally. The restriction of the over-expression phenotype to regions of *Hand1* expression also confirms observations from previous studies that indicated Hand1 plays a cell-autonomous role in the developing heart (Riley et al., 2000).

There is variation in the severity of the *Hand1* over-expression phenotype, with a range of hypermorphic phenotypes being observed from unaffected to severely affected. In the most severely affected embryos, the outflow tract is approximately twice the length of wildtype controls, and the presumptive ventricle is small, densely packed with cells and has no discernible lumen or trabeculae. In the moderately affected embryos, the outflow tract is elongated to a lesser extent, being around 50% longer than wildtype controls, and the ventricle is reduced in size but otherwise developing normally with the development of trabeculae and a clearly defined lumen. In a third class of over-expression phenotype only the presumptive ventricle is affected having a similar phenotype to the ventricle in severely affected embryos but lacking the outflow tract defect. Additionally, some transheterozygote embryos appear to be unaffected. The range in phenotype is most likely directly due to the observed variation in transactivator expression levels and pattern (see section 4.3.1.3). In the *Tet-Off-Hand1* strain, the transactivator displays a similar range of expression levels, from barely detectable to very strong, particularly in the outflow tract. As with the loss of function studies described in the previous chapter, it will be interesting to see if the removal of the PGKneo^r cassette from the *Tet-Off-Hand1* strain yields more robust transactivator expression, and what corresponding effect this may have on the levels of *Hand* over-expression and the resultant phenotype in transheterozygote embryos.

There are a greater number of cells in *Hand1* over-expression embryos, as a result the outflow tract is extended and the ventricle is densely packed. Therefore, it was

determined by immunofluorescence on embryonic sections whether this increase in cell number was due to elevated levels of proliferation, or reduced levels of apoptosis. The levels of apoptosis were not altered in over-expression embryos indicating that the increase in cell number is not caused by a lack of programmed cell death. However, it was demonstrated that the outflow tract and presumptive ventricle of *Hand1* over-expression embryos display enhanced levels of proliferation compared to wildtype controls, suggesting that *Hand1* over-expression induces cells to continue proliferating.

Given the increase in the levels of proliferation in the outflow tract and presumptive left ventricle of the *Hand1* over-expressing embryos, it was expected that there would be a concomitant decrease in the extent of cardiomyocyte differentiation. Accordingly, it was demonstrated by *in situ* hybridisation and quantitative real-time PCR that markers of cardiomyocyte differentiation were indeed down-regulated in *Hand1* over-expressing embryos. *Wnt11*, *Mlc2v* and *ANF* are all associated with cardiomyocyte differentiation, and *in situ* hybridisation revealed that all three markers were down-regulated in the affected areas of the heart tube. Furthermore, *ANF* and other cardiac differentiation markers *Chisel*, *GATA4* and *Nkx2.5* were shown to be significantly down-regulated by quantitative real-time PCR. Additionally, *in situ* hybridisation on frontal sections through *Hand1* over-expression embryos revealed that the 'extra', proliferative cells within the outflow tract were *Nkx2.5* negative, and so may be more like immature precursor cells. Together, these observations imply that there is a defect in cardiomyocyte differentiation in *Hand1* over-expressing embryos, and therefore, suggest that Hand1 may play a role in controlling cardiomyocyte proliferation versus differentiation. The parallel studies carried out in *Hand1*-null and *Hand1* over-expressing ES cell lines (see Chapter 6) lend support to this observation that Hand1 is involved in regulating the balance between cardiomyocyte differentiation and proliferation.

It seems, therefore, that endogenous Hand1 is involved in maintaining the correct balance between differentiation and proliferation in the regions of the heart in which *Hand1* is expressed. Consequently, appropriate *Hand1* expression is required for correct cell number in the developing heart. This role is reflected in the *Hand1* over-expressing embryos where cell number was seen to be greater in the ventricle and outflow tract than wildtype, and also in the tetraploid rescued *Hand1*-null embryos

where the presumptive left ventricle was thin-walled and the outflow tract was hypoplastic (Riley et al., 1998).

In zebrafish *hands off* mutants fewer cardiac precursors differentiate and those that do fail to fuse at the midline (Yelon et al., 2000)(see section 1.2.8). This has been attributed to failure of the anterior lateral mesoderm to expand, implicating *hands off* in proliferation of the anterior lateral mesoderm (Schoenebeck and Yelon, 2004) and also in determining the proper number of myocardial cell precursors (Trinh et al., 2005).

Myocardium is added to the outflow tract from the AHF in a process known as myocardialisation (see section 1.1.3.3.1). *Hand1* is not expressed in the cells of the anterior heart field, but it is expressed in the myocardium of the outflow tract. The cells that migrate from the AHF into the embryonic heart are undifferentiated, and are thought to terminally differentiate into cardiomyocytes once they arrive at their destination in the developing heart (Waldo et al., 2001). It is, therefore, a possibility that Hand1 is controlling this differentiation process in the outflow tract myocardium, balancing levels of proliferation or differentiation to maintain appropriate outflow tract remodelling. In the event of *Hand1* over-expression in the outflow tract, the balance shifts towards continued proliferation, resulting in increased cell number, abnormal outflow tract development and subsequent morphogenesis of the heart. The extension of the heart tube observed at ~E8.0 suggests that over-expression of *Hand1* exerts this proliferative effect on the cells arising from the primary heart field in addition to those migrating in from the anterior heart field.

ANF and *Chisel* are considered markers of secondary myocardium. Secondary myocardium has a distinct molecular profile that is different to primary myocardium, and once this program is initiated, secondary myocardium is thought to balloon out from the outer curvature of the primary heart tube to form the four chambers of the heart (see section 1.1.3.5). The down-regulation of *ANF* and *Chisel* in *Hand1* over-expressing embryos may, therefore, be responsible for the failure of the ventricle to balloon out, as differentiation into secondary myocardium is not initiated. Again, it appears possible that Hand1 may be controlling the balance between differentiation and proliferation in the presumptive left ventricle. In the event of *Hand1* over-expression in the presumptive left ventricle, as in the outflow tract, the balance shifts towards proliferation resulting in an increased number of cells and less differentiation with down-regulation of cardiac differentiation markers. Conversely, in tetraploid-rescued

Hand1-null embryos, the thin myocardial wall of the left ventricle in the absence of Hand1 could be caused by a shift in balance towards decreased proliferation, rather than secondary effects as previously thought (see section 1.2.6.4; (Riley et al., 2000)).

However, the lack of ventricular expansion when *Hand1* is over-expressed is inconsistent with the observation made by Togi et al. (2004) that Hand1 is required for chamber expansion, and although they too found that *ANF* and *Chisel* were downstream of Hand1, their expression was up-regulated in the event of *Hand1* over-expression. These conflicting results could be due to the differences in experimental approach. Togi and co-workers (2004) have placed *Hand1* under the control of the *Mlc2v* locus and, therefore, express *Hand1* throughout the entire ventricular region (see section 1.2.6.8). The altered expression domains could account for the differences observed between their model and this tet-inducible model. Additionally, *ANF* expression was down-regulated in the cardiac-specific knock-out of *Hand1* (see section 1.2.6.9; (McFadden et al., 2005), and *ANF* has since been identified as a downstream target for Hand1 in transcriptional assays (Morin et al., 2005). These studies indicate that Hand1 induces *ANF* expression, yet in *Hand1* over-expressing embryos, *ANF* is seen to be down-regulated by both *in situ* hybridisation and quantitative real-time PCR. However, the observed down-regulation of *ANF* in the ventricle and outflow tract following *Hand1* over-expression is an indicator of reduced cardiomyocyte differentiation not a direct transcriptional effect of Hand1.

Alternatively, it is a possibility that some aspects of the *Hand1* over-expression phenotype are caused by altered haemodynamics within the developing heart tube. Correct haemodynamics are known to be vital for cardiac morphogenesis to proceed normally (see section 1.1.3.4). Therefore, it is conceivable that the ventricular ballooning defect is a consequence of increased haemodynamic load brought about by elongation of the outflow tract and abnormal looping. However, due to the mosaic nature of transactivator expression, areas of the heart tube also appear, in some cases, to be affected in relative isolation. In particular, the fact that a few embryos are affected in the left ventricle only, without any outflow tract defect, whilst in others both the ventricle and outflow tract are affected, argues against significant haemodynamic involvement in generating the ventricular defect. Nonetheless, it is still likely to contribute to the phenotype in the severely affected embryos where the lumen is greatly reduced in size.

The levels of *Hand2* expression were not altered in *Hand1* over-expression embryos as shown by quantitative real-time PCR. Hand1 is thought to negatively regulate *Hand2* since *Hand2* is down-regulated when *Hand1* is mis-expressed in the right ventricle (Togi et al., 2004). In this tet-inducible gain of function model, however, the zone of *Hand1* expression is not enlarged; the level of *Hand1* expression is elevated exclusively in *Hand1*-expressing cells. The areas of predominant *Hand2* expression are not affected and alterations to *Hand2* expression are, therefore, not expected.

Real-time PCR on transheterozygote embryos also revealed that in addition to *Hand2*, the levels of *Mef2c* were also unchanged. Given the putative role of these two transcription factors in regulating differentiation of the AHF and its derivatives, this result provided evidence that the AHF is not affected in *Hand1* over-expressing embryos, and supports the assessment that the outflow tract elongation is not caused by increased recruitment of cardiomyocytes from the AHF.

It would appear that a large proportion of the embryos that over-express *Hand1* survive to term. So far, transheterozygote mice have been born at expected Mendelian frequencies, although, the total number looked at is relatively small. It seems unlikely that the most severely affected embryos could survive. Given that the severely affected only comprise a small fraction, 17 %, of the transheterozygote embryos examined, it is perhaps not surprising that there is not a significant decrease in the number of transheterozygotes born in those litters examined to date.

However, the large majority of *Hand1* over-expression embryos that do appear to survive to term presumably recover and continue to develop as normal; this is conceivable, as the moderately affected embryos do not have grossly abnormal hearts. *Hand1* expression in the heart peaks at E10.5 and then declines sharply. Perhaps as Hand1 levels decline after this time point, the heart is able to recover sufficiently so that by E12.5 and E14.5 it appears normal. It is thought that apoptosis might be involved in remodelling the outflow tract (Sharma et al., 2004) and this could account for the recovery of the elongated outflow tract. Additionally, the left ventricle is reduced in size in *Hand1* cardiac specific knock-out embryos (see section 1.2.6.9)(McFadden et al., 2005), but this also appears to recover by birth.

The survival and apparently normal phenotype of transheterozygote embryos and mice is also indicative that other lineages in which *Hand1* is expressed are not greatly

affected. It may be that the levels of transactivator expression are not high so *Hand1* expression is not elevated in these regions, or at least not sufficient to significantly alter morphogenesis. The possible defect in placental development in the event of *Hand1* over-expression does not appear to be as severe as the *Hand1*-null placental defect, as it does not appear to be detrimental to embryo survival. Nonetheless, the placental defect requires further investigation, in particular the placentae of severely affected embryos. However, the apparent hypoplasia in the placenta in the event of moderate levels of *Hand1* over-expression could be a result of increased Mash2 inhibition and consequently, a failure to maintain an appropriate spongiotrophoblast layer. It would also be interesting to examine the placentae further for confirmation of this putative mechanism, and also to investigate whether there are any alterations in trophoblast giant cell number since Hand1 is known to induce giant cell differentiation (Cross et al., 1995).

Cardiovascular defects can be linked to, and caused by, placental defects. It is, therefore, a possibility that the *Hand1* over-expression cardiac phenotype discussed in this chapter may be a secondary effect of a placental defect. However, the cell autonomous effects and specific alterations to cardiomyocyte proliferation and differentiation observed in this model argue against this possibility.

In summary, it appears that Hand1 is directly involved in controlling the balance between cardiomyocyte differentiation and proliferation. The *in vivo* gain of function study carried out here implies that over-expression of *Hand1*, exclusively in *Hand1* expressing cells at mid-gestation, causes increased proliferation at the expense of cardiomyocyte differentiation in the outflow tract and presumptive left ventricle, resulting in failure to maintain normal cardiac morphogenesis. Conversely, in *Hand1*-null embryos, there is an apparent failure to maintain an adequate pool of undifferentiated cardiomyocyte precursors leading to organ hypoplasia. This implicates Hand1 in the aetiology of human congenital heart defects since hypoplastic left heart and outflow tract defects are among the most common defects described in CHD (see section 1.1.2). Furthermore, cell-autonomous regulation by Hand1 of cardiomyocyte precursor addition during elongation of the heart tube has provided some insight into the process of directing cardiac progenitor cells along a path toward integrated myocardium. This is of particular relevance to cell-based therapies for cardiovascular disease.

The corresponding studies of cardiomyocyte differentiation in ES cell lines *in vitro*, which will be discussed in more detail in the following chapter, both support and add to the *in vivo* over-expression data, confirming that Hand1 plays a role in determining cardiomyocyte fate.

Chapter 6: The effect of Hand1 on cardiomyocyte differentiation *in vitro*

6.1 Introduction

When ES cells are differentiated *in vitro*, clonal cells proliferate and differentiate forming floating three-dimensional aggregates called embryoid bodies. Embryoid bodies have the potential to form all the specialised cell types of the developing embryo, including beating cardiomyocytes (see section 1.3). ES cells can also be manipulated genetically in order to study gene function. Therefore, ES cells provide a physiologically relevant model system in which to study the effect of loss and of over-expression of *Hand1* on cardiomyocyte differentiation *in vitro*. Moreover, these *in vitro* studies compliment the *in vivo* studies described in Chapters 4 and 5. *Hand1*-null ES cells were already available in house generated previously by successive rounds of gene targeting (Riley et al., 2000). It was, therefore, undertaken to generate a complimentary ES cell line that precociously over-expressed *Hand1*. These cell lines could then be analysed and compared to investigate the role of Hand1 in cardiomyocyte differentiation.

6.1.1 A role for Hand1 in cardiomyocyte differentiation

Tetraploid rescued *Hand1*-null embryos do form a linear heart tube, but the heart fails to loop and the myocardial wall of the ventricle is thin (Riley et al., 1998). Nevertheless, the formation of a heart tube demonstrated that cardiomyocyte differentiation can occur in the absence of *Hand1*. Furthermore, *Hand1*-null ES cells are capable of differentiating into beating cardiomyocytes and express cardiac specific transcripts (see section 1.2.6.4)(Riley et al., 2000). This again implies that Hand1 is not essential for differentiation of cardiomyocytes. However, it was noted that *Hand1*-null ES cells generate more cardiomyocytes than the wildtype R1 ES cell line suggesting that Hand1 may still be required in some form to regulate cardiomyocyte differentiation.

Hand1 is known to induce differentiation in extra-embryonic lineages, notably directing diploid trophoblast cells towards a trophoblast giant cell fate as has been demonstrated by over-expression of *Hand1* in the Rcho-1 cell line (Cross et al., 1995; Scott et al.,

2000). Additionally, *Hand1*-null trophoblast stem (TS) cells are deficient in trophoblast giant cell differentiation (Hemberger et al., 2004). Furthermore, Hand1 is thought to play a role in neural crest cell differentiation (see section 1.2.6.6; (Riley et al., 2000)).

In the developing embryo, *Hand1* is expressed at an early stage in a variety of mesodermal cell populations. It is expressed in the lateral plate mesoderm and its derivatives, including the cardiac mesoderm, and also in extra-embryonic mesoderm. Hand1 may, therefore, act as a 'gate-keeper' for the differentiation of the mesodermal populations in which it is expressed. In the absence of *Hand1*, in the *Hand1*-null ES cells, cells proceed along the default mesoderm pathway that is differentiation into cardiomyocytes. However, in the wildtype situation, Hand1 may either direct cells down alternative differentiation pathway(s), or directly block cardiomyocyte differentiation. This putative role for Hand1 was examined *in vitro* in *Hand1*-null and *Hand1* over-expressing ES cell lines.

6.2 Methods

6.2.1 Cell culture

All ES cell lines were maintained as described in section 3.2.1.5. The ES cell lines that were used are *Hand1*-null (Riley et al., 2000), *Hand1* heterozygote (Riley et al., 1998), R1 (ATCC catalogue number SCRC-1011), and *Tre2-Hand1* clone 39 transgenic line – a 'wildtype' ES cell line that carries the silent *Tre2-Hand1* transgene, one of the ES cell clones used for the generation of transgenic mice strains (see section 2.3.3.1). When included in the culture media, dox was added at 2 μg/ml.

6.2.2 Creation of stable ES cell lines

Tre2-Hand1 clone 39 (TG39) ES cells were co-transfected with a Tet-Off transactivator construct under the control of the TK promoter (pBIG3r; (Strathdee et al., 1999) and a pTK-Hyg selection plasmid (Clontech) using Effectene™ (Qiagen) (see section 3.2.2). Stable transformants were selected for as described in section 3.2.13. DNA was extracted (see section 2.2.6) and the stable clones were genotyped by PCR (see section 2.2.9) for the Tet-Off transactivator (primers 357 and 358, 900 bp and 357 and 575 150 bp), and *tubulin* (primers 694 and 695, 320 bp) as a control for loading. For primer sequences see Appendix 2. ES clones that were identified as stable transformants containing the Tet-Off transactivator were designated TG39tTA.

6.2.3 Characterisation of stable transfectants

The stable TG39tTA ES clones were grown up as ES cells and harvested. Poly (A) RNA (see section 3.2.6.2) and protein (see section 3.2.11) were extracted and RT-PCR (see sections 3.2.8-10), northern blotting (see section 3.2.7) and western blotting (see section 3.2.12) were performed. For RT-PCR, primers specific to *Tre2-Hand1* (675 and 676, 400 bp) Tet-Off transactivator (357 and 575, 150 bp) and *tubulin* (694 and 695, 320 bp) were used. See Appendix 2 for full primer sequences. For northern blotting, a *Hand1* specific probe was used (see section 3.3.2.1.1). For western blotting, antibodies to Hand1 (Santa Cruz), Cyclin D2 (AbCam), CDK4 (Santa Cruz) and GAPDH (Chemicon) were used. See Appenidx 4 for full antibody details. Densitometry was performed using the Scion Image Program as described in section 2.2.5.

6.2.4 *In vitro* differentiation of ES cell lines

ES cell lines were differentiated *in vitro* as described previously (see section 3.2.14). Samples were harvested at two-day intervals. Floating and adherent EBs were collected, washed twice in DEPC-PBS or PBS (for RNA and protein respectively) and the pellets snap frozen. Poly (A) RNA and protein were extracted from the samples as described in section 3.2.6.2 and section 3.2.11 respectively.

6.2.5 Estimating percentages of beating foci in embryoid bodies

The ES cell lines were *in vitro* differentiated as in section 3.2.14. Around 100 embryoid bodies were sampled, at random, at day 8, 10, 12 and 14 of differentiation. Beating foci were counted and calculated as a percentage of the total sample.

6.2.6 Single cell preparations of cardiomyocytes

Single cardiomyocytes were isolated from *in vitro* differentiated ES cells according to the protocol described by Maltsev et al. (1993).

ES cells were *in vitro* differentiated into embryoid bodies as normal (see section 3.2.14), until day four of differentiation when they were plated onto 0.1% gelatin coated tissue culture dishes. Five beating areas were mechanically isolated with a pipette tip at day 10 and day 14 and washed in Low Calcium medium (see Appendix 1). The isolated cells were spun down and resuspended in enzyme medium (see Appendix 1) for 30 minutes at 37°C in order to dissociate the individual cells. The cells were then

incubated in KB medium (see Appendix 1), with gentle shaking at room temperature, for one hour to complete dissociation. The dissociated cells were resuspended well in normal ES culture medium supplemented with 20% FCS and incubated overnight at 37°C, under normal cell culture conditions (see section 3.2.1.5).

6.2.7 Immunofluorescent staining of cells

Immunofluorescent staining of cells was performed with the MF20 (Developmental Studies Hybridoma Bank) and Nkx2.5 (Santa Cruz) antibodies (see Appendix 4).

Isolated cells plated onto 0.1% gelatin coated glass coverslips (13 mm diameter) were washed twice in PBS, and then fixed in 100% methanol for 10 minutes on ice. The coverslips were washed three times in PBS then transferred to microscope slides and circled with an Immedge wax pen. The cells were permeabilised with 0.5% Triton X-100/PBS for five minutes and blocked in 1% BSA/PBS (block) for 30 minutes. The slides were incubated with the primary antibody at the appropriate dilution (see Appendix 4) in block in a humidity chamber overnight at 4°C. A secondary antibody only coverslip was included as a control, and was left overnight in block. The cells were washed in block three times, for five minutes each. The slides were then incubated with the filtered secondary antibody, again at the appropriate concentration diluted in block (see Appendix 1), in a humidity chamber at room temperature for two to three hours. The slides were washed as above. Nuclei were stained, cells were mounted with coverslips and fluorescence was visualised as described in section 3.2.15.

6.2.8 Quantitative real-time PCR

Quantitative real-time PCR analyses were performed by Laurent Dupays, NIMR. Embryoid bodies were harvested at day 10 and day 14 of differentiation as described in section 6.2.4. Total RNA was extracted using Trizol reagent according to the manufacturer's protocol (see section 3.2.6.1) and quantitative real-time PCR was performed as described in section 4.2.14. Primers specific for *Hand1*, *MLC2v*, *α-cardiac actin*, *Nkx2.5*, *ANF*, *GATA-4*, *Chisel*, *Mef2c*, *Irx4*, *Hand2*, *Brachyury*, *eomes*, *Fgfr1* and *GATA1* were used. Primer sequences are listed in Appendix 3.

6.3 Results

6.3.1 Generation of stable *Tre2-Hand1* ES cell lines with a Tet-Off transactivator

Tre2-Hand1 clone 39 (TG39) ES cells were transfected with a Tet-Off transactivator construct, in which transactivator expression is under the control of the TK promoter, known to be active in ES cells. A TK-hygromycin selection plasmid (Clontech) was co-transfected for selection of stable transformants. The transfection and selection process was carried out twice. From the first round of hygromycin selection, 74 ES cell clones were picked, and from the second round of selection, 38 ES cell clones were picked. The selected clones were expanded in culture, DNA was extracted and the clones were genotyped by PCR for the Tet-Off transactivator. Two PCR reactions were carried out, one for the full-length transactivator and one for a smaller fragment for confirmation. From the first set of clones, two ES cell lines were identified as carrying the transactivator (Figure 6.1). A further four clones were identified as possible stable transformants that required confirmation (Figure 6.1), and from the second set of clones 13 transactivator positive ES cell lines were identified (not shown).

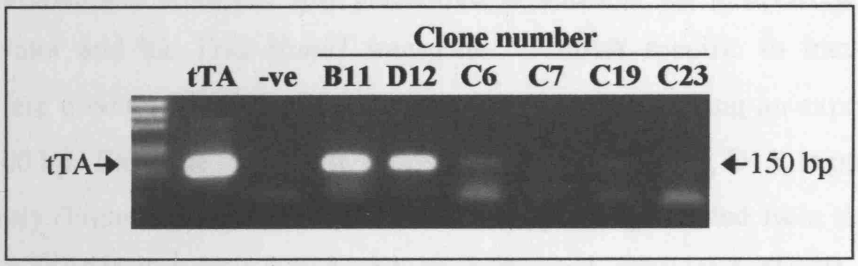


Figure 6.1. PCR analysis to determine Tet-Off transactivator stable transfectants. TG39 ES cells were stably transfected with a Tet-Off transactivator construct under the control of a TK promoter, and stable transformants were selected for hygromycin resistance. DNA was extracted from resistant clones and PCR was performed using primers specific for the Tet-Off transactivator. A Tet-Off construct was included as a positive control. Two stable clones, B11 and D12, were shown to contain the Tet-Off transactivator; four clones, C6, C7, C19 and C23 required further confirmation.

6.3.2 Characterisation of the stable ES cell lines

6.3.2.1 Two stable cell lines express the *Tre2-Hand1* transgene transcript in ES cells

As discussed in section 1.2.6.2, Hand1 is both necessary and sufficient for trophoblast differentiation. Hand1 is also required for the very early differentiation of trophectoderm in the developing blastocyst (Cross et al., 1995). The polar trophectoderm overlies the proliferating, pluripotent inner cell mass, which produces signals to block the effects of differentiation inducing factors, such as Hand1. ES cells are derived from the inner cell mass and therefore likely produce similar repressive signals. Indeed Oct3/4 are thought to be vital for maintaining ES cell pluripotent capacity (Niwa et al., 2000). Loss of Oct3/4 in ES cells leads to rapid induction of *Hand1* and differentiation into trophectoderm (Niwa et al., 2000). Thus, ES cells may inherently contain factors that block *Hand1* expression.

Therefore, having identified stable transformants, it was important to ensure that the cell lines were expressing *Hand1*. Poly (A) RNA was extracted from the stable TG39tTA ES cell clones and RT-PCR was performed to confirm the expression of both the transactivator and the *Tre2-Hand1* transgene. Primers specific to transgenic *Tre2-Hand1* were used that span the β-globin poly(A) fragment having an expected product size of 400 bp. From the first set of TG39tTA clones, one clone, D12, expressed *Hand1* prematurely (Figure 6.2A). Since D12 was the only line generated from the first round of ES clone selection that genotyped correctly and expressed *Hand1* correctly, it prompted the second round of ES clone selection. It was imperative to create a second line to ensure that any altered characteristics observed in the D12 line were consistent and occurred as a direct result of *Hand1* over-expression, and were not secondary to the site of transactivator integration. The second set of clones was tested in the same way as described above to check for *Tre2-Hand1* expression. From the second set of clones, again only one line out of 13 was shown to express *Hand1* in ES cells by RT-PCR, clone 11 (Figure 6.2B). For two of the lines the results were inconclusive due to insufficient starting material as shown by a failure to detect *tubulin* expression.

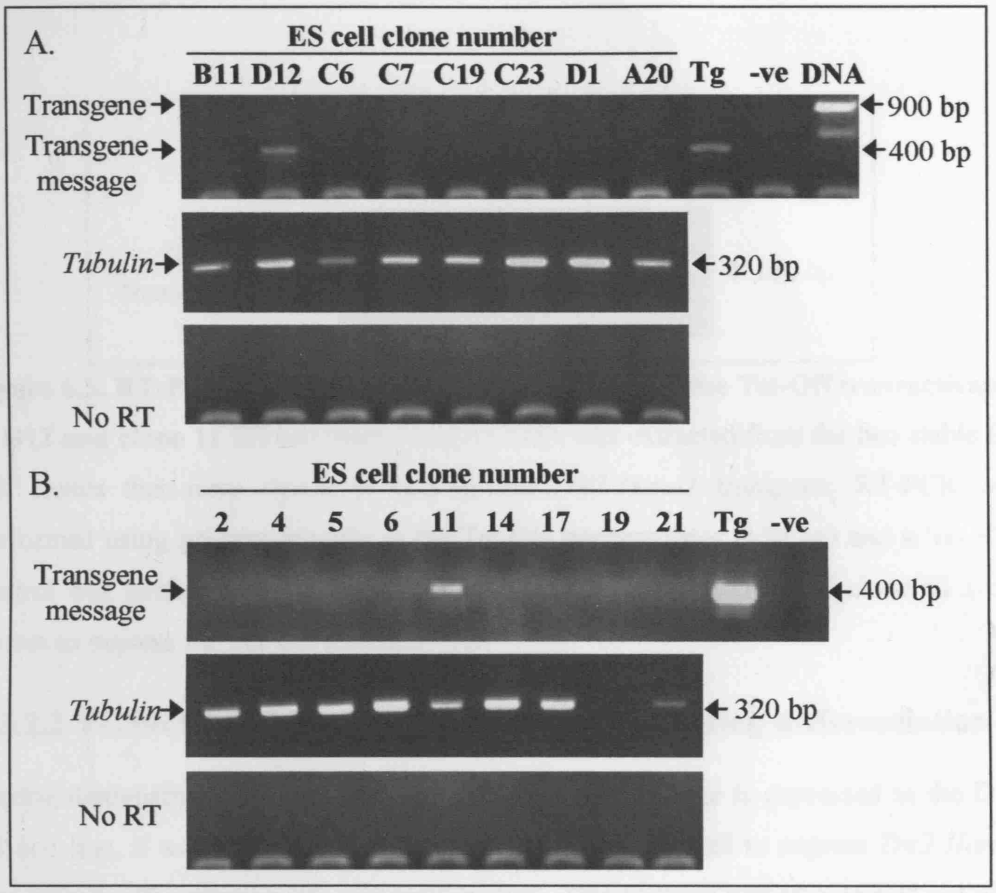


Figure 6.2. RT-PCR analysis to determine expression of the *Tre2-Hand1* transgene in TG39tTA stable clones. Poly(A) RNA was extracted from the two sets of TG39tTA stable ES cell clones, two transactivator negative ES cell clones (D1 and A20) were included as negative controls and 293 Tet-Off™ cells transfected with the *Tre2-Hand1* transgenic construct were included as a positive control. RT-PCR was performed using primers specific for *Tre2-Hand1* transgene message (400 bp) and for *tubulin* (320 bp), and a 'no RT' control was also performed. (A) *Tre2-Hand1* expression was only observed in one ES cell line, D12, from the first set of clones, which prompted the generation of a second set of clones, (B), from which one clone, clone 11, was shown to express the *Tre2-Hand1* transgene.

Additionally, both the D12 and clone 11 derived cell lines were shown to express the Tet-Off transactivator by RT-PCR (Figure 6.3).

In conclusion, two stable ES cell clones were shown to express the *Tre2-Hand1* transgene correctly, D12 and clone 11. However, as clone 11 was only recently identified it has not been characterised further (see Discussion).

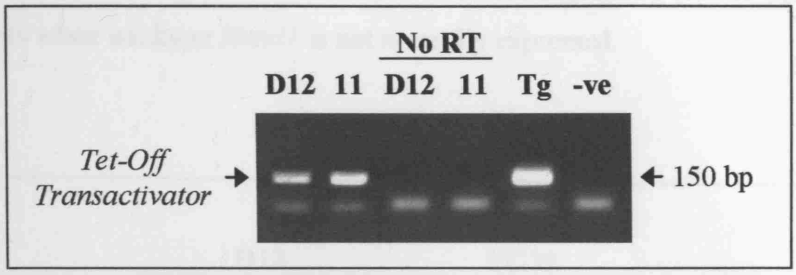


Figure 6.3. RT-PCR analysis to determine expression of the Tet-Off transactivator in D12 and clone 11 ES cell lines. Poly(A) RNA was extracted from the two stable ES cell clones that were shown to express the *Tre2-Hand1* transgene, RT-PCR was performed using primers specific to the Tet-Off transactivator (150 bp) and a 'no RT' control was performed. Positive control as Figure 6.2. Both D12 and clone 11 were shown to express the Tet-Off transactivator.

6.3.2.2 TG39tTA cell lines express *Tre2-Hand1* during differentiation

Having demonstrated that the *Tre2-Hand1* transgene message is expressed in the D12 ES cell line, it was then confirmed that the cell line continued to express *Tre2-Hand1* throughout differentiation into embryoid bodies. This analysis was also carried out with a view to obtaining some insight into the extent of over-expression of *Hand1* at stages when wildtype *Hand1* is also expressed. Normally, *Hand1* expression is not detected until around day 4 of differentiation in embryoid bodies at which point expression is relatively weak, whilst *Hand1* expression peaks at around day 10 of differentiation, declining thereafter (N. Smart, personal communication).

The D12 cell line and the parent control cell line, TG39, were *in vitro* differentiated and embryoid bodies were harvested at day 2, 4, 6 and 8. Poly(A) RNA was extracted and RT-PCR was performed for *Tre2-Hand1* as described above. In addition to expression in ES cells (day 0), *Tre2-Hand1* expression was observed in D12 embryoid bodies at day 2 and 4 of differentiation (Figure 6.4). Unfortunately, the poly(A) RNA samples for day 6 and 8 D12 embryoid bodies appeared to be degraded, as shown by the failure to detect *tubulin* expression (Figure 6.4). In contrast, the TG39 control cell line displayed no expression of the *Tre2-Hand1* transgene throughout differentiation.

The D12 cell line, by revealing *Tre2-Hand1* expression in ES cells and during the early stages of embryoid body differentiation, is therefore expressing *Hand1* precociously at time points when wildtype *Hand1* is not normally expressed.

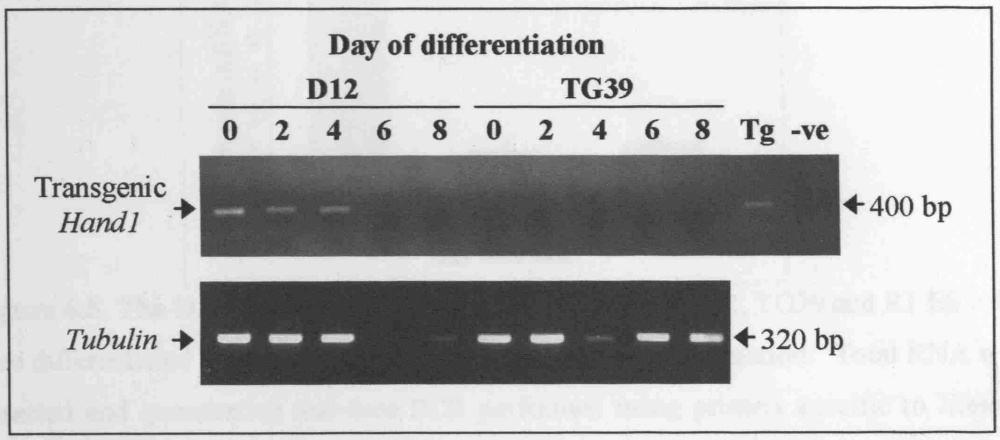


Figure 6.4. RT-PCR analysis to determine *Tre2-Hand1* expression in the D12 ES cell line throughout differentiation. D12 and TG39 ES cells were *in vitro* differentiated into embryoid bodies and harvested at day 2, 4, 6 and 8 of differentiation. Poly(A) RNA was extracted and RT-PCR performed using primers specific to *Tre2-Hand1* and *tubulin*. Positive control for *Tre2-Hand1* message as in Figure 6.2. *Tre2-Hand1* expression was observed in the D12 line at day 2 and day 4 of differentiation. It was not observed at day 6 and 8, however this is likely due to an insufficient amount of cDNA in these samples as shown by the failure to detect *tubulin* expression. The parent TG39 cell line does not express the *Tre2-Hand1* transgene at any timepoint throughout differentiation.

6.3.2.3 *Hand1* is over-expressed in the D12 cell line

As quantitative real-time PCR is a more sensitive technique, it was employed to quantify the extent of *Hand1* over-expression in the D12 cell line in a more precise manner. In order to carry out this analysis, total RNA was extracted from embryoid bodies from the four cell lines harvested at day 10 of differentiation, and quantitative real-time PCR was performed for *Hand1* expression. In the D12 cell line, *Hand1* is expressed between five and six fold compared to the control parent cell line, TG39 (Figure 6.5). In addition, it was shown that the two control cell lines, TG39 and R1, have fairly comparable levels of *Hand1* expression (Figure 6.5). The real-time PCR

result confirmed that, as expected, the D12 cell line does over-express *Hand1* during differentiation into embryoid bodies.

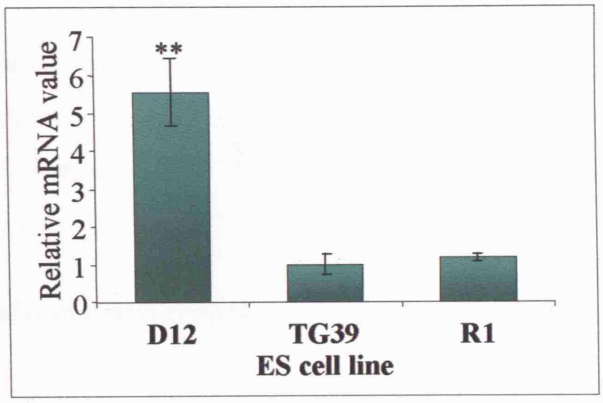


Figure 6.5. The D12 ES cell line over-expresses *Hand1*. D12, TG39 and R1 ES cells were differentiated *in vitro* and harvested at day 10 of differentiation. Total RNA was extracted and quantitative real-time PCR performed using primers specific to *Hand1*. The D12 ES cell line was shown to express *Hand1* five- to six-fold higher than both R1 wildtype and TG39 control ES cell lines. Furthermore, the R1 and TG39 cell lines were shown to have equivalent levels of *Hand1* expression. ** p=0.05

6.3.2.4 *Tre2-Hand1* protein expression

In addition to assessing transgene message expression in the over-expression cell lines, it was attempted to confirm the premature expression of Hand1 protein from the *Tre2-Hand1* transgene and also evaluate the level of Hand1 protein expression at subsequent time points. Samples were harvested from the D12 and TG39 ES cells, and from *in vitro* differentiated embryoid bodies at day 2, 4, 6 and 8 of differentiation. Protein was extracted and western blotting was performed using a Hand1 antibody (Santa Cruz). Unfortunately, no protein expression was detected in the D12 cell line at the ES cell stage or throughout differentiation. However, the expected wildtype expression normally seen at day 6 and 8 was also not detected (not shown). The positive control was detected, albeit weakly, and the TG39 parent cell line was shown to express Hand1 at day 6 and 8, indicating the western blotting procedure and antibody were functioning correctly. This indicates that the D12 samples may have degraded (see Discussion).

6.3.3 Analysis of Hand1 function during ES cell differentiation *in vitro*

For all subsequent analysis, only the D12 cell line has been tested. The D12 line was generated first; therefore, the analysis of Hand1 function began prior to obtaining the second line derived from clone 11. As mentioned previously, this second line remains largely uncharacterised.

6.3.3.1 Hand1 affects differentiation of ES cells into cardiomyocytes *in vitro*

From previous unquantified observations, the *Hand1*-null ES cells appeared to form more beating foci of coupled cardiomyocytes than R1 wildtype ES cells when differentiated into embryoid bodies *in vitro*. Based on this observation, it became of interest to discover what the effect on cardiomyocyte differentiation would be if *Hand1* expression was induced at earlier time points, and subsequently at higher levels than wildtype.

Therefore, having established an ES cell line that precociously over-expressed *Hand1*, this line was assessed in comparison to the parent TG39 line (ES cell line that carries a stable integration of the *Tre2-Hand1* transgene that is silent in the absence of any transactivator). Furthermore, to compare the effects of over-expression to loss of function, the *Hand1*-null ES cell line was assessed in comparison to the R1 wildtype line. It was important only to compare the *Hand1*-null cells with the R1 and the D12 with the TG39 so as to compare like for like, to allow for inherent differences between the two distinct control cell lines (R1 and TG39).

Beating cardiomyocytes are normally first observed at around day 8 of differentiation *in vitro*. The four ES cell lines were differentiated *in vitro* and beating foci were counted at day 8, 10, 12 and 14 of differentiation. It was found that, overall, the *Hand1*-null cells formed more beating cardiomyocytes than R1, and that the D12 line formed fewer beating cardiomyocytes than TG39 (Figure 6.6). At day 10, the *Hand1*-null cells formed around five-fold more cardiomyocytes and by day 14 there was a two-fold increase in the *Hand1*-null cells. In the D12 line, no cardiomyocytes were observed until day 14, at which point there was around a 14-fold reduction in the number of cardiomyocytes. In addition to a change in the number of beating foci frequency, it was

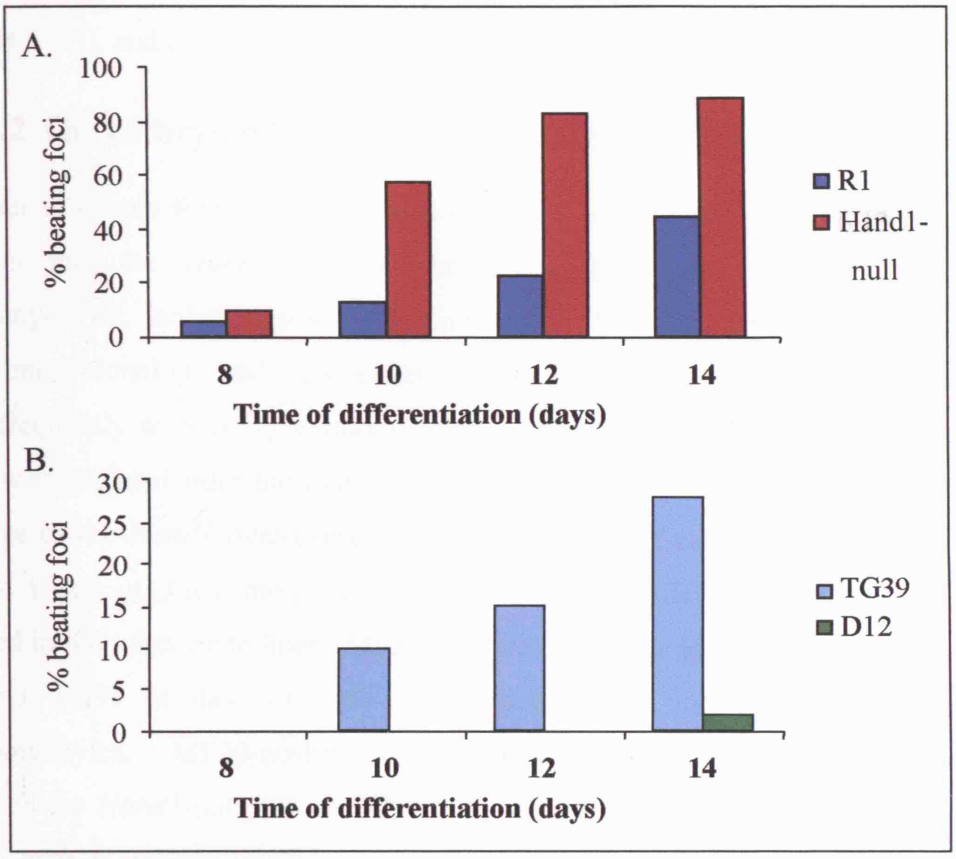


Figure 6.6. Hand1 dosage affects cardiomyocyte differentiation. *Hand1* over-expressing (D12), *Hand1*-null, R1 and TG39 wildtype ES cells were differentiated into floating embryoid bodies *in vitro* over a time course up to day 14. Around 100 embryoid bodies were sampled from each cell line at two-day intervals, beginning at day 8 of differentiation. Beating foci were counted and calculated as a percentage of the total sample. (A) *Hand1*-null ES cells differentiate into cardiomyocytes earlier and at greater frequency than R1 wildtype cells. (B) *Hand1* over-expressing ES cells (D12) differentiate into cardiomyocytes later, and at a lower frequency than TG39 control.

also noticeable that the pattern of cardiomyocyte differentiation in *Hand1*-null cells and the D12 was dramatically altered. In the *Hand1*-null cells there was a steep increase in the number of beating foci at day 10 which subsequently began to level out, whereas the R1 cells continued to differentiate into beating cardiomyocytes at a steady rate throughout the time course examined (Figure 6.6A). The TG39 cells formed fewer beating foci overall than the R1 line, but the pattern was comparable, with the number of beating foci increasing steadily. However, in the D12 line there was a considerable

delay in differentiation as there were no beating cardiomyocytes observed until day 14 (Figure 6.6B), and even then this was a rare occurrence.

6.3.3.2 The embryoid body beating foci are cardiomyocytes

In order to ensure that the beating foci observed were indeed cardiomyocytes, and to confirm that the *Hand1* over-expressing line (D12) was deficient in forming cardiomyocytes, isolated cells were immunostained for sarcomeric myosin using the MF20 monoclonal antibody (Developmental Studies Hybridoma Bank), which has been used frequently to identify cardiomyocytes (Riley et al., 2000; Waldo et al., 2001). Cells were isolated from the four cell lines at day 10 and day 14 of differentiation. In the case of the *Hand1* over-expressing cell line, areas of cells that were isolated were chosen based on their morphological/phenotypical similarities to the beating areas isolated in the other three lines. MF20-positive cells were identified in the *Hand1*-null, R1 and TG39 at day 10 and day 14 confirming that the beating foci were cardiomyocytes. MF20-positive cells isolated at day 10 representative of those observed for *Hand1*-null, R1 and TG39 ES cell lines are shown in Figure 6.7. In the *Hand1* over-expressing line however, there were no cells positive for MF20, implying that cardiomyocyte differentiation is impaired by elevated levels of *Hand1* expression. Immunofluorescence was also attempted using an Nkx2.5 antibody for a final confirmation of cardiomyocyte identity. However, the Nkx2.5 antibody displayed high levels of background activity preventing proper analysis (not shown).

6.3.3.3 Alterations in cardiac specific gene expression

In order to quantify changes in gene expression between the *Hand1*-null and *Hand1*-over-expressing cell lines, real-time PCR was performed for a variety of cardiomyocyte markers. *Hand1*-null, *Hand1*-over-expressing and the two wildtype, R1 and TG39, ES cell lines were differentiated *in vitro* and embryoid bodies harvested at day 10 of differentiation, since this was when the greatest difference in numbers of cardiomyocytes was observed (see section 6.3.3.1) and *Hand1* expression is at it's highest. Total RNA was extracted and used for real-time analysis.

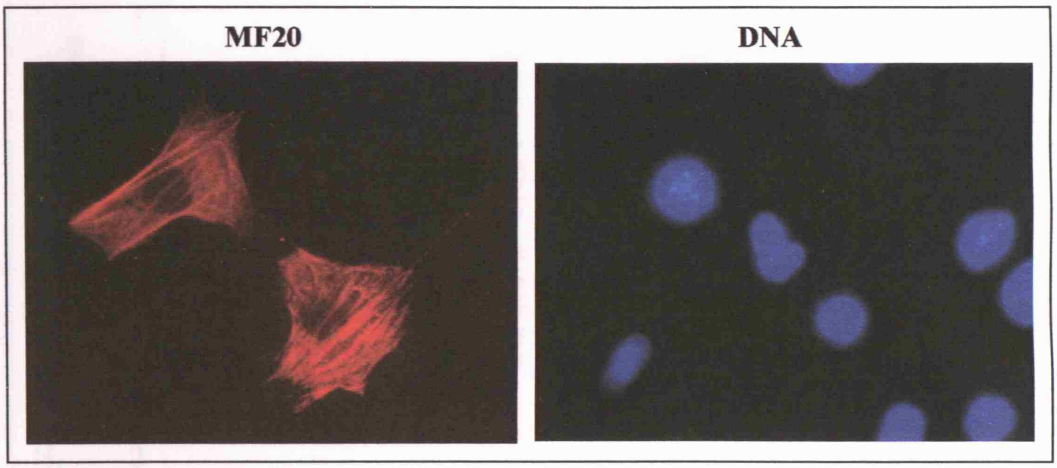


Figure 6.7. Embryoid body beating foci comprise bona fide cardiomyocytes. Single cells were isolated from beating areas of day 10 of differentiated *Hand1*-null, *Hand1* over-expressing, R1 and TG39 ES cells. Cardiomyocytes were identified by immunofluoresence on isolated cells using the MF20 antibody (Developmental Studies Hybridoma Bank) that marks myocardial sarcomeric myosin, and cells were stained with bis-benzimide to visualise nuclei. MF20-positive cardiomyocytes, such as those depicted in this Figure, were identified from the *Hand1*-null, R1 and TG39 cell lines, confirming that the beating foci represent cardiomyocytes.

Nkx2.5 is one of the earliest markers of myocardial differentiation (Komuro and Izumo, 1993; Lints et al., 1993). *α-cardiac actin* (*α-CA*) is a pan-cardiomyocyte marker (Sassoon et al., 1988; Lyons et al., 1991) and additionally, has been identified as being up-regulated in the *Hand1*-null embryoid bodies in a previous RDA screen (Smart et al., 2002). Significantly, therefore, *Nkx2.5* and *α-CA* were shown to be dramatically down-regulated in the *Hand1*-over-expressing D12 cell line and up-regulated in the *Hand1*-null cell line (Figure 6.8). These results confirm the previous observations that when differentiated *in vitro*, the *Hand1*-null ES cell line generates an increased number of cardiomyocytes, and the *Hand1* over-expressing cells generate fewer cardiomyocytes.

Other markers of cardiomyocyte differentiation were also analysed. *GATA4* is an indicator of early myocardial fate, particularly for posterior regions of the heart tube, but is also expressed in other lineages including primitive endoderm (Arceci et al., 1993). *ANF* is considered a marker of working myocardium, a more differentiated population of cardiomyocytes (Christoffels et al., 2000). *Mlc2v* and *Irx4* are markers of

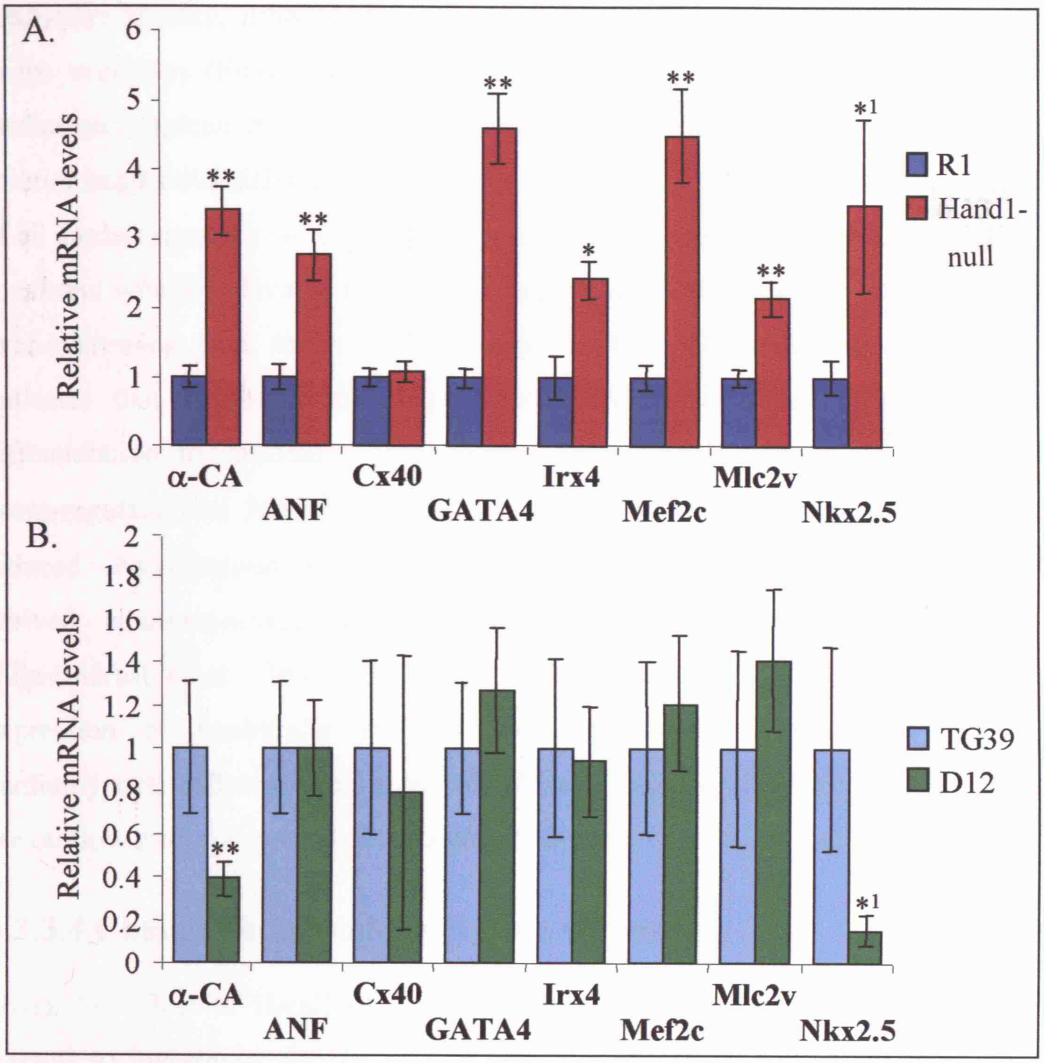


Figure 6.8. Quantitative real-time PCR analyses to assess the effect of Hand1 on cardiac gene expression. *Hand1*-null, *Hand1* over-expressing, R1 and TG39 ES cells were differentiated *in vitro* and harvested at day 10 of differentiation. Total RNA was extracted and used for real-time PCR analysis for an array of cardiac markers. (A) *Hand1*-null and R1 cell lines were compared. All cardiac markers except the gap junction protein Connexin 40 (Cx40), including *α-cardiac actin* (*α-CA*) and *Nkx2.5* were found to be up-regulated in the *Hand1*-null cell line. (B) *Hand1* over-expressing and TG39 cell lines were compared. *α-cardiac actin* (*α-CA*) and *Nkx2.5* were found to be down-regulated in the *Hand1* over-expressing cell line, but other cardiac markers were expressed at levels equivalent to wildtype expression levels. ** $p<0.05$, * $p<0.07$, *1 $p<0.2$

ventricular identity, although their expression in the developing embryo is not restricted to the ventricles (Franco et al., 1999; Bruneau et al., 2000). Mef2c is involved in cardiac and skeletal muscle differentiation, and in particular thought to be critical for anterior heart field differentiation (Dodou et al., 2004; Von, I et al., 2004). The levels of all cardiac markers were significantly increased in the *Hand1*-null line (Figure 6.8), consistent with the elevated numbers of cardiomyocytes in this line. In the D12 *Hand1* over-expressing line, these cardiac markers were not altered significantly, which indicates that in the event of precocious *Hand1* over-expression, a degree of differentiation to cardiomyocytes does occur, although considering the significant down-regulation of *Nkx2.5* and *α-CA*, commitment to the cardiac lineage is much reduced. As discussed previously in section 1.3.2, it has been suggested that ES cell derived cardiomyocytes more closely resemble the embryonic outflow tract (Fijnvandraat et al., 2003a; Fijnvandraat et al., 2003b). Therefore, given that the expression of ventricular markers appeared to be normal, the reduction in cardiomyocyte differentiation in the *Hand1* over-expressing cells could be attributed to the outflow tract component of embryoid body derived cardiomyocytes.

6.3.3.4 Changes in mesodermal gene expression

Given the effect of Hand1 on cardiomyocyte differentiation *in vitro*, it was also of interest to investigate the theory that Hand1 may act as a putative 'gatekeeper' for differentiation of mesodermal populations (see section 6.1.1), specifically to investigate which other mesodermal lineages are differentiating at the expense of cardiomyocytes following *Hand1* over-expression, and equally which other lineages are deficient in a *Hand1*-null background.

Quantitative real-time PCR was, therefore, also performed to examine the expression of several mesodermal genes, including the general mesoderm marker Brachyury (T), FGF receptor 1 (*Fgfr1*), the T-box transcription factor eomesodermin (*eomes*) and GATA1. *Fgfr1* and *eomes* are expressed in mesodermal populations but have also been shown to have an effect on cardiomyocyte differentiation. *Fgfr1*-null ES cells have been shown to differentiate into fewer cardiomyocytes than wildtype *in vitro* (Dell'Era et al., 2003), and recently, a putative role for eomes has been identified in regulating ventricular size in *Xenopus* (Ryan et al., 2004). GATA1 is essential for normal haematopoietic development, in particular lateral plate mesoderm derived erythrocytes (Pevny et al., 1991; Fujiwara et al., 1996) and thus, represents and alternative mesodermal fate.

GATA1 levels were not altered in any of the four cell lines examined (see Figure 6.9) suggesting that Hand1 does not affect erythroid development or other aspects of haematopoiesis in which GATA1 is intricately involved. Interestingly, *eomes* was seen to be up-regulated in the *Hand1*-null embryoid bodies, and down-regulated in the *Hand1* over-expressing embryoid bodies. Inhibition of eomes in *Xenopus* embryos results in the development of small hearts, with a hypoplastic ventricle and outflow tract, whilst over-expression of eomes results in enlarged, hyperplastic hearts (Ryan et al., 2004). This led to the hypothesis that eomes inhibits differentiation and promotes continued proliferation of cardiomyocytes. The real-time PCR result for *eomes* was, therefore, unexpected. From the observations in *Xenopus* described above that suggest eomes inhibits differentiation of cardiomyocytes, it might be predicted that *eomes* would be reduced in the *Hand1*-null cell line that appears to have elevated levels of differentiation, and increased in the over-expressing cell line that appears to have lower levels of differentiation (see Discussion). *Fgfr1* expression was down-regulated in the *Hand1* over-expressing cell line, but unchanged in the *Hand1*-null cell line. *Fgfr1*-null ES cells differentiate into fewer beating cardiomyocytes than wildtype ES cells (Dell'Era et al., 2003). Therefore, it might be expected that *Fgfr1* expression is reduced when *Hand1* is over-expressed and fewer cardiomyocytes differentiate. It might, therefore, follow that it is up-regulated in the *Hand1*-null cell line, however *Fgfr1* expression appears to be unaffected.

Brachyury (T) is essential for mesoderm differentiation and as such, is a general marker for early mesodermal populations, but is not expressed in the heart (Ryan and Chin, 2003). It is somewhat surprising, therefore, that *brachyury* expression appears to be down-regulated in both the *Hand1*-null and *Hand1* over-expressing cell lines (see Discussion).

6.3.3.5 Hand1 influences cell cycle regulation

The altered levels and rates of differentiation in the *Hand1*-null and *Hand1* over-expressing cell lines suggested there may be an associated alteration of proliferation in these cell lines, as proliferation and differentiation are inversely correlated. Furthermore, in *Hand1*-over-expressing embryos there is elevated proliferation in the outflow tract and presumptive left ventricle, accompanied by a decrease in differentiation (see Chapter 5). It follows, therefore, that in the event of *Hand1* over-

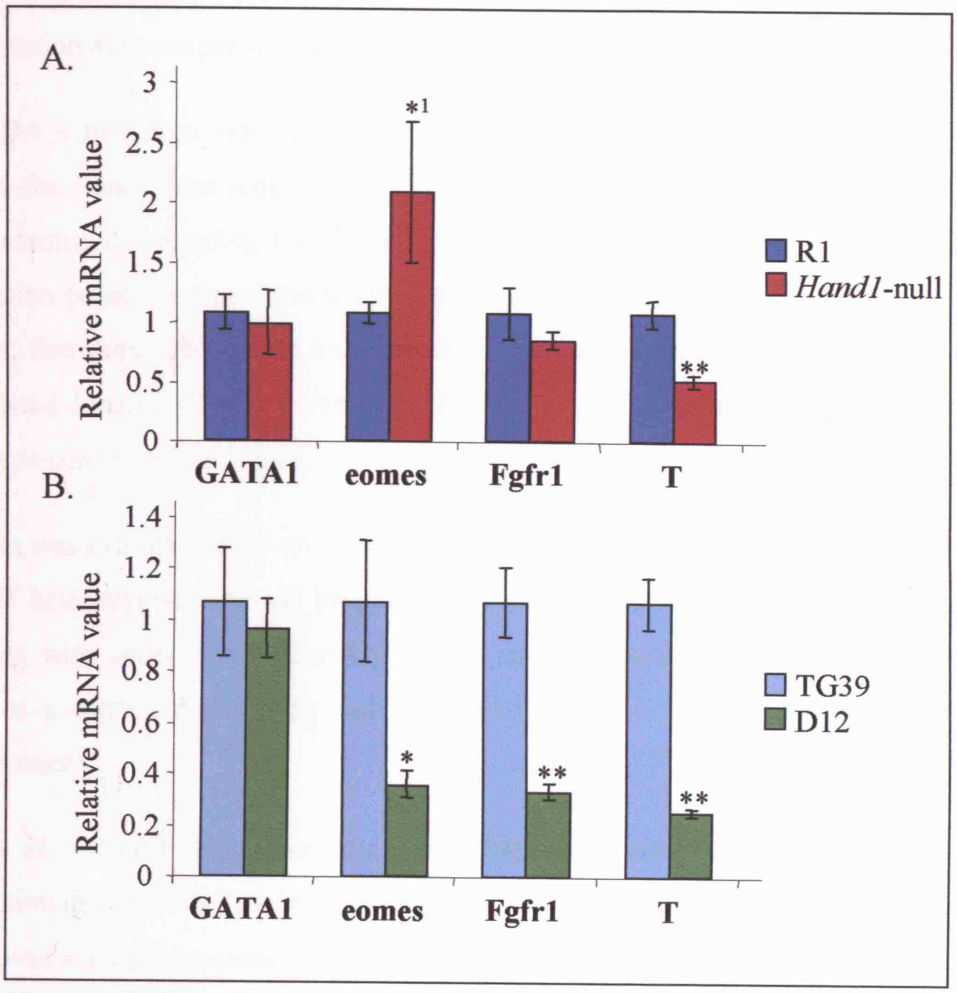


Figure 6.9. Quantitative real-time PCR analyses to assess the effect of Hand1 on mesodermal gene expression. *Hand1*-null, *Hand1* over-expressing, R1 and TG39 ES cells were differentiated *in vitro* and quantitative real-time PCR performed as Figure 6.8 for mesodermal marker genes *GATA1*, *eomesodermin* (*eomes*), *Fgfr1* and *brachyury* (T). (A) *Hand1*-null and R1 cell lines were compared. *GATA1* and *Fgfr1* expression levels were unchanged. *Eomes* expression was elevated in the *Hand1*-null cell line, and *T* expression levels were reduced. (B) *Hand1* over-expressing and TG39 cell lines were compared. GATA1 levels were unchanged. *Eomes*, *Fgfr1* and *T* expression levels were down-regulated in the *Hand1* over-expressing cell line. ** $p<0.05$, *$p<0.1$, *[1]$p<0.2$

expression, there may be a reduction in exit from the cell cycle, resulting in continued proliferation at the expense of differentiation.

An RDA screen was carried out previously in order to identify putative downstream targets for Hand1 (see section 1.2.6.7; (Smart et al., 2002)). Several cell cycle genes were identified, including Cyclin D2, which is involved in regulating the G1/S phase restriction point, the transition from Gap 1 (G1) to DNA synthesis (S) in the cell cycle. It was, therefore, decided to examine the levels of expression of Cyclin D2 and it's associated kinase, CDK4, in the *Hand1*-null, *Hand1*-over-expression and respective wildtype/control ES cell lines.

Protein was extracted from embryoid bodies from the four ES cell lines, and also from a *Hand1* heterozygote ES cell line, at day 14 of differentiation, and used for western blotting with antibodies against Cyclin D2 and cognate kinase, CDK4. GAPDH was used as a control for loading and for quantification of protein expression levels by densitometry.

In the *Hand1*-null cells there was a two-fold reduction in Cyclin D2 and three-fold reduction in CDK4 protein levels. Conversely, in the D12 *Hand1* over-expressing cells, there was a 3.5-fold increase in Cyclin D2 and 2.5-fold increase in CDK4 (Figure 6.10). This suggests that Hand1 is upstream of Cyclin D2, and also shows that changes in Cyclin D2 protein levels are reflected in relative CDK4 protein levels. Cyclin D2 and CDK4 regulate entry into the cell cycle by controlling the G1/S phase transition. It appears that *Hand1*-null cells are prevented from progressing through the cell cycle as Cyclin D2 and CDK4 levels are reduced and consequently exit the cell cycle prematurely, inducing differentiation. Whereas, the increased Cyclin D2 and CDK4 levels enables the *Hand1* over-expressing cells to remain cyclic and continue to proliferate, at the expense of differentiation.

6.4 Discussion

Following the observation that *Hand1*-null ES cells differentiated into more beating cardiomyocytes than their wildtype R1 counterpart, and the previously published studies indicating that Hand1 induces differentiation of the Rcho-1 cell line (Cross et al., 1995; Scott et al., 2000) and of TS cells (Hemberger et al., 2004) into trophoblast giant cells, the role of Hand1 in cardiomyocyte differentiation was investigated. Furthermore,

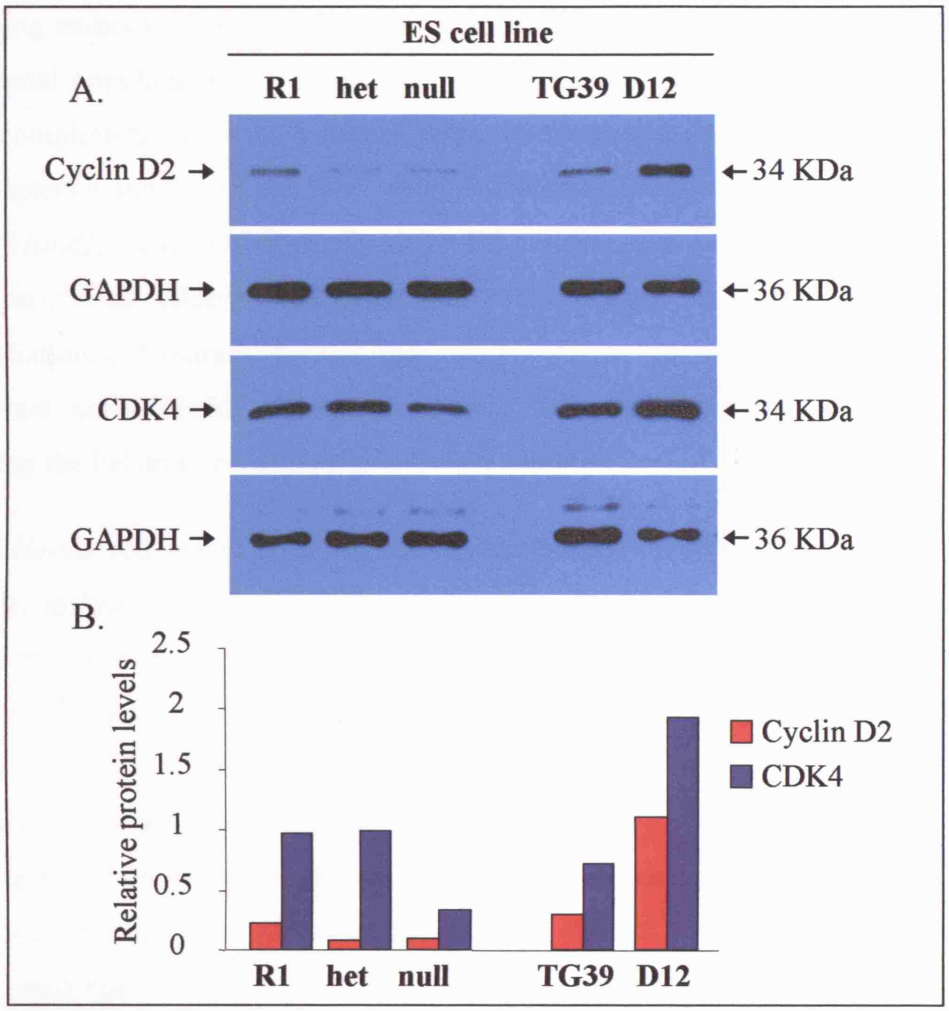


Figure 6.10. Hand1 influences cell cycle progression mediated by Cyclin D2 and CDK4. *Hand1*-null, *Hand1* heterozygote (het) *Hand1* over-expressing (D12), R1 wildtype and TG39 control ES cells were differentiated *in vitro*, harvested at day 14 of differentiation, protein extracted and western blotting performed using antibodies to Cyclin D2 (AbCam), CDK4 (Santa Cruz) and GAPDH (Chemicon) (A). Relative protein expression levels were quantified by densitometry (B). In the *Hand1*-null cells, Cyclin D2 and CDK4 protein levels are reduced two- and three-fold respectively compared to R1. In the *Hand1* over-expressing cell line, Cyclin D2 and CDK4 levels are elevated 3.5- and 2.5-fold respectively compared to TG39.

given that *Hand1* is expressed in a variety of mesodermal cell types within the developing embryo, Hand1 may play a more general role in directing differentiation of mesodermal populations. In order to investigate this putative role for Hand1 further, and to compliment the *in vivo* studies performed with the tet-inducible *Hand1* model (see Chapters 4 and 5), ES cell lines were generated that precociously express and over-express *Hand1*. A *Hand1* over-expression ES cell line was characterised and analysed in comparison to wildtype and *Hand1*-null ES cell lines in terms of cardiomyocyte differentiation. Contrary to the role of Hand1 in promoting differentiation of trophoblast cells, Hand1 appears to block differentiation of cardiomyocytes by regulating the balance between differentiation and proliferation.

A *Tre2-Hand1* cell line (TG39) that was created during the generation of the transgenic responder mouse lines (*Tre2-Hand1*; see section 2.3.3.1) was stably transfected with a Tet-Off transactivator construct, under the control of the TK promoter, known to be active in ES cells. Two cell lines, D12 and clone 11, were successfully generated that expressed *Hand1* in ES cells as shown by RT-PCR. Since the D12 ES cell line was generated before the second line, the D12 cell line has been characterised further. The D12 line has been shown to express *Hand1* precociously in ES cells and throughout differentiation into embryoid bodies by RT-PCR. Quantitative real-time PCR analysis has revealed that at day 10 of differentiation, the D12 line expresses *Hand1* at greatly elevated levels compared to the TG39 control ES line, between five- and six-fold higher.

Unfortunately, no Hand1 protein has been detected yet, at any point throughout the time course. There was a failure to detect the wildtype levels of Hand1 protein at day 6 and 8, which perhaps indicates poor sample integrity. Given that there is an effect on the differentiation of the D12 *Hand1* over-expressing cell line from both beating foci and gene expression pattern perspectives, an assumption would be that the Hand1 protein is being expressed early and/or at greater levels. However, this remains to be confirmed.

Having now generated the second over-expression line, clone 11, this cell line needs to be assessed in the same way as the D12 cell line to ensure appropriate expression of the *Tre2-Hand1* transgene throughout differentiation in terms of both message and protein, and also to estimate the degree of *Hand1* over-expression in this cell line. This second line will also rule out any possible effects of transactivator integration site in the D12 line. Additionally, both cell lines must be cultured in the presence of dox to show that

inhibiting *Tre2-Hand1* transgene expression restores the wildtype characteristics of the TG39 parent cell line i.e that differentiation proceeds normally in these cell lines when *Hand1* is not over-expressed.

Hand1 clearly has an effect on ES cell differentiation into cardiomyocytes *in vitro*. In the absence of Hand1, *Hand1*-null ES cells form a great number of cardiomyocytes compared to the R1 wildtype ES cell line. Moreover, most *Hand1*-null cardiomyocytes appear to differentiate earlier, at day 10, after which cardiomyocyte numbers do not fluctuate enormously, whereas in the R1 wildtype line cardiomyocyte differentiation progresses at a steady rate with the highest numbers observed at day 14, the latest time point analysed. Therefore, a lack of Hand1 seems to result in increased and accelerated cardiomyocyte differentiation.

Conversely, in the event of *Hand1* over-expression in the D12 cell line, far fewer cardiomyocytes are formed than in the comparative control cell line TG39. Furthermore, in the D12 over-expression cell line cardiomyocytes are generally not observed until day 14, long after the first cardiomyocytes are formed in the TG39 control cell line. Therefore, over-expression of *Hand1* appears to result in decreased and significantly delayed cardiomyocyte differentiation.

These observations were supported by the real-time PCR analysis that showed that markers of cardiomyocyte differentiation, *Nkx2.5* and *α-CA*, were down-regulated in the D12 over-expression line and up-regulated in the *Hand1*-null cells. *α-CA* is known to be further downstream than Hand1, and the respective increase and decrease in *α-CA* expression was expected. *Nkx2.5* is thought to lie upstream of Hand1 since *Nkx2.5*-null embryos fail to express *Hand1* in the presumptive left ventricle (Biben and Harvey, 1997). Therefore, the observation that Hand1 affects the levels of *Nkx2.5* expression suggests that Hand1 can feedback to modify *Nkx2.5* expression levels.

Various other cardiac markers were examined by quantitative real-time PCR analyses. In the *Hand1*-null cell line, expression of all cardiac markers was increased. This most likely reflects the enormous increase in the number of beating cardiomyocytes generated by this line. The *Hand1* over-expressing cell line, however, displayed no alterations in cardiac gene expression, except *Nkx2.5* and *α-CA*, already mentioned above. This suggests that, although beating cardiomyocytes are not observed, commitment to a cardiac fate is occurring since *Mef2c*, *GATA4*, *Irx4* and *Mlc2v* are all

expressed at levels comparable with wildtype control cell lines. In the case of *GATA4* and *Mef2c*, the unaltered levels of expression in the *Hand1* over-expressing line potentially also reflects their expression in other non-cardiac lineages, such as endoderm (Arceci et al., 1993) and skeletal muscle (Edmondson et al., 1994), respectively. *Irx4* and *Mlc2v* are used as markers for ventricular cardiomyocyte differentiation (O'Brien et al., 1993; Bruneau et al., 2000). The normal levels of *Irx4* and *Mlc2v* expression observed in the *Hand1* over-expressing line suggests that progression towards ventricular identity may be unaffected in the event of *Hand1* over-expression in this study. *Irx4* and *Mlc2v* are expressed in other regions but relatively weakly compared to the predominant expression in the ventricles; expression in other lineages is unlikely to account for their normal expression in *Hand1* over-expressing embryoid bodies. Therefore, if the ventricular markers are expressed at normal levels in the *Hand1* over-expressing cell line, it is possible that the great reduction observed in cardiomyocyte differentiation and the down regulation of *Nkx2.5* and *α-CA* is attributable to loss of the outflow tract component. This is especially feasible given the recent study that implied that ES cell-derived cardiomyocytes exhibit characteristics of outflow tract and early chamber myocardium ((Fijnvandraat et al., 2003a; Fijnvandraat et al., 2003b); see section 1.3.2).

The altered level and rate of cardiomyocyte differentiation in the *Hand1*-null and *Hand1* over-expressing cell lines is indicative of alterations in proliferation. Furthermore, as discussed in the previous chapter, there is an increase in proliferation in *Hand1* over-expressing embryos. Elevated levels of proliferation are indicative of aberrant cell cycle regulation. Cyclin D2 and its associated cyclin-dependent kinase, CDK4, regulate entry into the cell cycle by controlling the transition from G1 (Gap 1) to S phase (DNA synthesis). Cyclin D2 is expressed in the developing heart (Kang et al., 1997), and has been shown to induce CDK4 activity, and also induces cardiomyocyte proliferation in primary cultures of neonatal rat ventricular myocytes (Busk et al., 2005). Additionally, Cyclin D2 was one of the cell cycle regulator genes that were identified as a putative downstream target of Hand1 in an RDA screen (see section 1.2.6.7; (Smart et al., 2002)). Given the above, it was decided to investigate the levels of Cyclin D2 and CDK4 protein in *Hand1*-null, *Hand1* heterozygote, *Hand1* over-expressing and wildtype differentiated ES cells by western blotting. It was found that in the *Hand1* over-expressing cell line, the reduced and delayed cardiomyocyte differentiation was associated with increased levels of Cyclin D2 and CDK4. The increased levels of

Cyclin D2 and CDK4 are, therefore, indicative of sustained entry into the cell cycle and consequently enhanced proliferation. The opposite was true in the *Hand1*-null cell line, where there appears to be down-regulation of Cyclin D2 and CDK4, indicating premature exit from the cell cycle and lower levels of proliferation, resulting in enhanced differentiation of cardiomyocytes.

This confirms that Cyclin D2 is downstream of Hand1, and that CDK4 is controlled in part by Cyclin D2, as this is reflected in the changes in CDK4 expression observed in the different cell lines. Furthermore, this implies that, at least *in vitro*, Hand1 may be involved in maintaining the correct balance between differentiation and proliferation of cardiomyocytes by regulating Cyclin D2 levels in the cell.

Moreover, given the observed increase in proliferation in the *Hand1* over-expressing embryos *in vivo*, Hand1 might also be involved in maintaining the balance between differentiation and proliferation, and consequently maintaining the correct number of cardiomyocytes, in the developing heart. Therefore, it seems likely that elevated levels of Cyclin D2 may be the cause of the increased cell number and elevated levels of proliferation observed in the *Hand1* over-expressing embryos, by stimulating the cells in the outflow tract and left ventricle to enter the cell cycle and remain proliferative.

Additionally, it has been demonstrated recently that Cyclin D2 represses hypertrophy by forcing cardiomyocytes through the cell cycle, resulting in cell division over hypertrophic growth, and it appears as though Cyclin D2 levels determine whether cells grow by hypertrophy or whether they proliferate (Busk et al., 2005). This is particularly interesting in light of the fact that *Hand1* down-regulation is associated with cardiac hypertrophy (see section 1.2.7; (Natarajan et al., 2001; Thattaliyath et al., 2002a)). The down-regulation of Hand1 may, therefore, result in down-regulation of Cyclin D2, which in turn may be a contributing factor towards hypertrophic growth.

It was further postulated that Hand1 may be involved in directing the differentiation of the various mesodermal populations in which it is expressed in the developing embryo, particularly in promoting differentiation along alternative pathways to a cardiomyocyte fate. As described above, in the absence of Hand1 it seems that, *in vitro*, differentiation into cardiomyocytes is the default pathway, and that in the event of *Hand1* over-expression there is reduced cardiomyocyte differentiation. It was, therefore, of interest to discover whether there were any changes in mesodermal gene expression in the

various ES cell lines. So far, only four markers have been analysed. GATA1 expression was determined to be normal in both *Hand1*-null and *Hand1* over-expressing embryoid bodies. GATA1 has been shown to be required for normal blood cell development during embryogenesis (Fujiwara et al., 2004). Blood cells, as part of the circulatory system, are derived from the lateral plate mesoderm, in which *Hand1* is expressed and, therefore, represent an alternative mesodermal fate to cardiomyocyte differentiation. However, Hand1 does not appear to have any effect on haematopoiesis. It has been shown that Hand1 is required for vascular refinement (see section 1.2.9) and blood vessels are also derived from the lateral plate mesoderm, it may therefore be more pertinent to determine whether there are any changes in vascular markers in the *Hand1*-null and *Hand1* over-expressing cell lines. Recently, an enhancer was identified in *Drosophila* that is responsible for both cardiovascular and haematopoietic *hand* expression (Han and Olson, 2005). Given the high level of transcriptional conservation in heart development, it has been suggested that vertebrate *Hand* genes may also be involved in haematopoietic development in addition to cardiogenesis (Han and Olson, 2005). It would be of interest, therefore, to continue examining the *Hand1*-null and *Hand1* over-expressing cell lines for any alterations in haematopoietic development.

Some extra-embryonic membranes (allantois, amnion, chorion and yolk sac) are also derived from the lateral plate mesoderm. *Hand1* is transiently expressed in extra-embryonic lineages, including yolk sac, amnion and allantois, and also in the trophectoderm where it has been shown to promote the differentiation of trophoblast giant cells (see sections 1.2.5.1 and 1.2.6.1). Since ES cells are derived from the inner cell mass of blastocysts, they do not tend to form extra-embryonic tissues. Therefore, if Hand1 promotes extra-embryonic differentiation and blocks cardiomyocyte differentiation, this may never be observed in the ES cell model generated in this project. However, loss of *Oct3/4* in ES cells induces de-differentiation into trophectoderm accompanied by an up-regulation of *Hand1* expression in ES cells (Niwa et al., 2000). It may be of interest, therefore, to investigate markers of trophectoderm in the *Hand1* over-expressing and *Hand1*-null cell lines. Alternatively, Hand1 may not be exclusively involved in controlling the differentiation of any cell lineage, but rather its role is to regulate the balance between cardiomyocyte proliferation and differentiation as described above.

Other mesodermal markers investigated by quantitative real-time PCR were *brachyury*, *eomes* and *Fgfr1*. Eomes and Fgfr1 have previously been shown to exert an effect on cardiomyocyte differentiation and so were not used to investigate alternate mesodermal fates. Excluding the down-regulation of *Fgfr1* in the *Hand1* over-expressing line, the real-time PCR results were unexpected. *Fgfr1*-null ES cells differentiate into fewer cardiomyocytes than wildtype *in vitro* (Dell'Era et al., 2003), and therefore its down-regulation in the *Hand1* over-expressing line where fewer cardiomyocytes differentiate is perhaps not surprising. However, it was expected that there would be an opposing effect in the *Hand1*-null line that was not observed. *Fgfr1* expression levels in *Hand1*-null embryoid bodies were normal. Similarly, the results for *eomes* expression were unexpected. Eomes has been shown to regulate heart size in *Xenopus* (Ryan et al., 2004) and from this study, Eomes has been predicted to inhibit differentiation and promote proliferation of cardiomyocytes. This is, therefore, a very similar role to that suggested for Hand1 by the results of this project. The fact that *eomes* is up-regulated when *Hand1* is absent and down-regulated when *Hand1* is over-expressed seems paradoxical. Maybe it is an attempt at a compensation mechanism to counter-act the effects of the forced alterations in *Hand1* expression.

Finally, *brachyury* expression was significantly down-regulated in both the *Hand1*-null and *Hand1* over-expressing cell lines. This suggests that mesoderm differentiation is impaired in both cell lines. This could be an indication that *Hand1* gene dosage may regulate mesodermal differentiation. However, in general, the efforts made to explore the theory that Hand1 acts as a gate-keeper for mesodermal cell differentiation have been inconclusive and require further investigation.

Chapter 7: General Discussion

A gain of function model for Hand1 has been successfully generated using the endogenously targeted *Tet-Off-Hand1* strain and the transgenic *Hand1* responder line, *Tet-Hand1*. When the *Tet-Hand1* responder is crossed with the *Tet-Off-Hand1* strain transheterozygotes embryos are generated that, at E9.5, over-express *Hand1* roughly two-fold above the level of wildtype *Hand1*, as shown by quantitative real-time PCR. In the most severely affected embryos, the distal outflow tract is hugely extended, looping is abnormal and the presumptive left ventricle is small, densely packed and has failed to expand out from the heart tube. The *Hand1* over-expression phenotype has been shown to be a result of over-proliferation, likely due to elevated levels of the G1/S phase regulator, Cyclin D2 and it's cognate kinase, CDK4, which is accompanied by a reduction in cardiomyocyte differentiation. Furthermore, studies in the *Hand1*-null and *Hand1* over-expressing ES cell lines have corroborated the results observed in the *in vivo* model. Therefore, it seems likely that Hand1 is involved in maintaining the equilibrium between proliferation and differentiation in the developing heart, and consequently ensuring the correct cell number, by regulating cardiomyocyte exit from the cell cycle (Figure 7.1). This is a previously unappreciated role for Hand1. However, Hand1 has been implicated in cell cycle control previously, as cell cycle regulator genes were identified as putative downstream targets for Hand1 (Smart et al., 2002), and moreover, *Hand1* has been shown to be down-regulated in gastric (Kaneda et al., 2002) and pancreatic cancers (Hagihara et al., 2004), which suggests Hand1 might be involved in maintaining appropriate cell cycle control. It would be of interest to make use of the gain of function model to evaluate this role for Hand1. It would also be of interest to examine in more detail the effects of this gain of function model in the placenta. Furthermore, there are other aspects of the *Hand1* over-expression phenotype that have not been discussed within, such as in severely affected embryos there is an increased distance between the developing forebrain and the first branchial arch and the first branchial arch is also reduced in size. *Hand1* is expressed in the medial region of the first branchial arches at the point of midline fusion; therefore, over-expression of *Hand1* in this region could be affecting branchial arch development, which could lead to the apparent hypoplasia of the first branchial arch and the

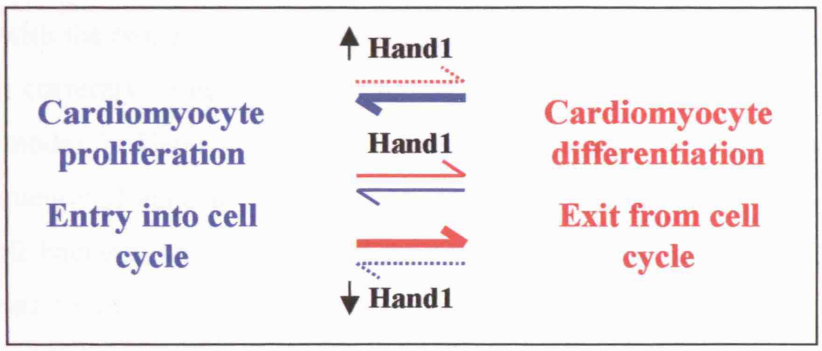


Figure 7.1. Proposed model of Hand1 function in the developing mouse heart. Hand1 is involved in maintaining the equilibrium between cardiomyocyte proliferation and differentiation in the developing mouse heart by regulating the balance between continued entry into, and exit from, the cell cycle. Changes in Hand1 dosage alter this balance, such that increased levels of Hand1 causes a shift towards cardiomyocyte proliferation, whilst decreased levels of Hand1 results in a shift towards cardiomyocyte differentiation.

increased distance to the forebrain. The gain of function model could be employed to investigate these and other potential roles for Hand1, however at present, this is hindered by the difficulty in generating sufficient numbers of severely affected transheterozygote embryos with the strains used so far. The two latest transgenic responder lines will be tested for expression levels, any further work on the gain of function model, therefore, depends on these two lines and the possibility of improving transactivator expression levels in the *Tet-Off-Hand1* strain by Cre excision of the PGKneo^r cassette (discussed in section 4.4). Consequently, efforts to improve the model are ongoing. One question in particular that remains to be answered is what happens to the extra cells in *Hand1* over-expressing embryos? Given that all but the most severely affected transheterozygote embryos appear to survive, it may be that there is some sort of compensation mechanism in place to ensure normal development. Embryos could be examined at immediately later stages, E10.5 and E11.5 to investigate what happens to the extra cells arising from the over-proliferation defect, for example by checking for increased levels of apoptosis in the outflow tract and left ventricle.

The use of the *Tet-Off-Hand1* and the *Tet-Hand1* strains to achieve rescue of the *Hand1*-null phenotype to generate the temporal and spatial knock-out of *Hand1* has proved unsuccessful so far, however the numbers examined up to this point are limited and, therefore, this avenue is by no means exhausted. In view of this, the *Tet-Hand1*

line, along with the two additional *Tre2-Hand1* transgenic lines that have been received recently, are currently being used in continued efforts to generate the tet-inducible loss of function model for Hand1. However, rescue is likely to be a rare event given the one in eight frequency of generating the appropriate genotype (compound heterozygotes on a *Hand1*-null background) and the variation in transactivator expression levels in the *Tet-Off-Hand1* strain.

Further efforts to generate the loss of function model and perhaps improve the gain of function model will involve crossing the *Tet-Off-Hand1* strain with a strain that ubiquitously expresses Cre recombinase, such as CMV-Cre (genOway), in order to excise the PGKneo^r cassette, as mentioned above. It is entirely possible that the presence of the PGKneo^r cassette in the *Tet-Off-Hand1* strain is contributing to the observed variation in transactivator expression levels. In the original publication on the knock-in of the tet system into the endothelin receptor B (*Ednrb*) locus by Shirley Tilghman's group, it was noted that in the responder strain the PGK promoter within an adopted PGK-hyg selection cassette inhibited the basal Tre2 promoter. In this instance the PGK-induced inhibition of transcription was fortuitous since it prevented leaky expression of *Ednrb*, from the responder, in the absence of transactivation (Shin et al., 1999). However, in our case the PGKneo^r may well be inhibiting the endogenous *Hand1* promoter, thus preventing appropriate expression of the transactivator. Removal of the cassette may, therefore, optimise expression of the transactivator, which hopefully in turn will improve the chances of rescuing the *Hand1*-null phenotype with the various strains already available. Moreover, optimisation of transactivator expression could also open up the potential for a variety of new studies. For example, the modified *Tet-Off-Hand1* strain could be used, in conjunction with appropriate responder strains, to examine the forced expression of any gene of interest exclusively in regions of *Hand1* expression, including putative downstream targets of Hand1. Generating strains like these might also enable attempts to be made at rescuing either the entire *Hand1*-null heart phenotype, or specific subsets of *Hand1*-null defects via tet-induced transgenic expression of potential downstream targets of Hand1.

If and when the tet-inducible *Hand1* system is able to rescue the *Hand1*-null phenotype and the precise kinetics of transactivator repression by dox have been ascertained, dox can be injected into pregnant mothers to switch off *Hand1* expression at specific timepoints in the 'rescued' compound heterozygote embryos. Initially efforts would be

focused on processes to which Hand1 function has previously been linked such as cardiac looping, ventricular expansion, outflow tract morphogenesis and cardiac neural crest migration (see section 1.2.6). For example, dox can be administered to pregnant mothers just prior to looping. Embryos can then be dissected at specific timepoints during and after cardiac looping and their precise molecular and cellular phenotype characterised in terms of potential cardiac defects. During cardiac looping, it will be particularly interesting to examine the expression of various cell adhesion and extra-cellular matrix molecules including *fibronectin*, *tenascin-X* and *laminin* since the extra-cellular matrix has been implicated in regulation of cell movement during cardiac looping (see section 1.1.3.4) and Hand1 has been implicated in organisation of the extra-cellular matrix and consequently in cell migration (see section 1.2.6.7). Similarly, to investigate the putative role for Hand1 in cardiac neural crest migration and differentiation in the outflow tract, dox can be administered at the appropriate time prior to crest migration into the outflow tract and embryos characterised for crest-derived defects, such as shortened outflow tract and deficient tube elongation (Waldo et al., 2005a). It would also be possible to isolate and culture neural tubes from wildtype and tet-induced *Hand1*-null embryos to compare the rate of neural crest migration, proliferation and differentiation. If generated, the tet-inducible knock-out of *Hand1* would enable continuing study of Hand1 function, not just within the developing heart, but also in the various other lineages in which *Hand1* is expressed including limbs, and as mentioned briefly above, the placenta and the branchial arches. Equally, should the modification to the *Tet-Off-Hand1* strain result in more efficient expression of the transactivator, over-expression of both *Hand1* and any other gene of interest could be assessed in other *Hand1* expressing lineages.

A *tet*-inducible knock-out model would also provide an opportunity to investigate a recently proposed, additional, function for Hand1; namely modifying cardiac responses in the myocardium to genetic insult or environmental stresses and mediating hypertrophic signalling (Hill and Riley, 2004). Thus far, Hand1 has not been directly linked with human congenital or adult-onset heart disease. This may be due to the fact that mutations in *Hand1* that result in a functional null are likely to be lethal *in utero* due to associated placentation defects. This does not preclude the existence of regulatory region mutations in important distal elements or association studies whereby Hand1 is indirectly implicated in heart disease. However, *Hand1* has been seen to be down-regulated in patients with cardiomyopathy (Natarajan et al., 2001) and in rodents

with induced hypertrophy (Thattaliyath et al., 2002a) and in a recent study on CLP-1 (cardiac lineage protein-1) -null mice, it was suggested that an observed down-regulation of *Hand1* in the hearts of CLP-1 embryos directly promotes a foetal form of cardiac hypertrophy (Huang et al., 2004). Furthermore, in mice deficient for the homeobox transcription factor Irx4, adult onset cardiomyopathy is preceded by significantly reduced expression of *Hand1* in the left ventricle at mid-gestation stages (Bruneau et al., 2001). Therefore, the *tet*-inducible model could be used to 'switch off' *Hand1* during ventricular maturation, for example by administering dox to pregnant mothers at E14.5, and then switch *Hand1* back 'on' again, by removing dox at E16.5, prior to investigating the resultant neonate/adult mice for any cardiac phenotype (Figure 7.2). This approach would address the question as to whether Hand1 is required in the heart during a key developmental window to ensure appropriate post-natal ventricular function.

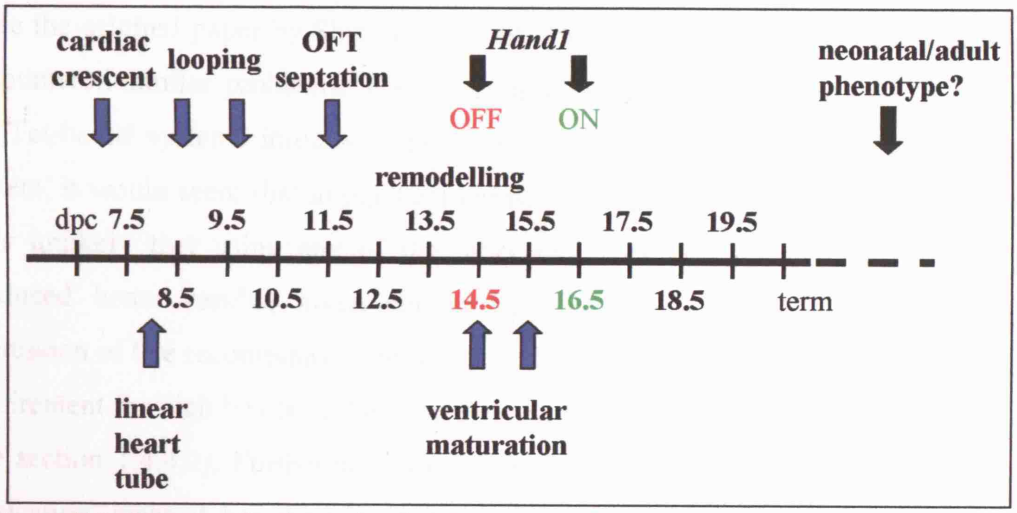


Figure 7.2. Strategy for investigating Hand1 embryonic function in mediating post-natal ventricular function. Timeline depicting the major stages of embryonic cardiac development. Using the tet-inducible knock-out of *Hand1*, *Hand1* expression can be 'switched off' for a two-day window during ventricular maturation, by administration of dox at E14.5 and removal at E16.5. Neonatal/adult mice will then be observed for any abnormal cardiac phenotype.Additionally, the majority of transheterozygote embryos that are over-expressing *Hand1*, albeit at relatively low levels, appear to survive without further complication. It may be worth observing transheterozygote mice until well into adulthood for any incidents of premature death and any signs of cardiovascular disease that may give insight into the role Hand1 plays in mediating cardiac hypertrophy.

Efforts are going to be continued to generate the loss of function model for Hand1 in order to gain further insight into both the function of Hand1 but also to elucidate the signals of cardiac morphogenesis and how these translate to the morphogenetic mechanisms involved in forming the adult heart, and further understanding of the aberrant morphogenetic processes that underlie cardiovascular and congenital heart disease.

However, it is possible, regardless of the ongoing efforts described above to optimise the tet-inducible *Hand1* system, that the system will be continually confounded by the caveats identified thus far, most notably mosaic and varying levels of transactivator expression. This will prevent the gain and loss of function models being used for further investigation into Hand1 function. It is interesting to note that there have been a limited number of examples published using a knock-in of the Tet-Off system *in vivo* since the original paper by Shin et al., 1999. This suggests that other groups may have encountered similar problems. Therefore, despite the great potential that was seen for the Tet-based systems initially in providing an easily controlled, reversible inducible system, it would seem that in practical terms, it proves far more difficult to implement. It is unlikely that using any of the other inducible systems available would have produced better results, given similar inherent problems of mosaic and ectopic expression of Cre recombinase (see section 1.4.4.2), and moreover an often pathological requirement for high levels of the inducer, such as the required high levels of tamoxifen (see section 1.4.4.2). Furthermore, the Tet-Off system is advantageous in its bimodal application, since it has the potential to enable both the 'switching on' and 'off' of *Hand1* at will, and analysis of gain and loss of function.

The ES cell model of Hand1 involvement in cardiomyocyte differentiation *in vitro* also has great potential for a variety of continued studies. It has already been shown that in *Hand1*-null ES cells *in vitro* cardiomyocyte differentiation is enhanced, and that *Hand1* over-expressing ES cells it is delayed, and that this is likely caused by Hand1 regulation of D-type Cyclins to control the balance between proliferation and differentiation. However, many cardiomyocyte characteristics have not been examined, including the frequency of embryoid body beating, which can be assessed easily by counting and then compared between the *Hand1*-null and *Hand1* over-expressing ES cell lines. The ES cell-derived cardiomyocytes can also be examined in terms of electrophysiological properties by patch clamp analysis. The patch clamping technique can be used on

isolated cardiomyocytes to determine the action potential of a cell, and the shape of the action potential can be used to distinguish between different functional cell types (see section 1.3.2)(Maltsev et al., 1993). Therefore, patch clamping could be performed on the *Hand1*-null and *Hand1* over-expressing cell lines to determine the predominant cardiomyocyte phenotypes produced by each cell line. It would be interesting to determine the phenotype of the *Hand1*-null cells that appear to exit the cell cycle and differentiate prematurely to see if they remain as fairly primitive pacemaker-like cardiomyocytes or proceed to terminal differentiation stages such as atrial- or ventricular-like. *Hand1*-null and *Hand1* over-expressing cardiomyocytes could also be examined more closely using electron microscopy and associated marker analysis for alterations in sarcomeric structure and expression of structural proteins. Information from patch clamp and ultrastructure analyses should provide an indication of the overall phenotype of the cardiomyocytes generated by the *Hand1*-null and *Hand1* over-expressing ES cell lines compared to wildtype, which in turn provides insight into the specific role of Hand1 at a cellular level in the developing heart. Additionally, the ES cell lines, and indeed *Hand1* over-expressing and tet-induced *Hand1*-null embryos should they be generated, could also be used to further the attempts already made at identifying downstream targets of Hand1 using microarray analysis.

Given the tet-inducible nature of the *Hand1* over-expressing cell line, *Hand1* expression can be manipulated with dox at any chosen time point. For instance, cells could be maintained with dox in the media until the point of maximal *Hand1* expression, around day 10 of differentiation (Smart et al., 2002), so that cardiomyocyte differentiation progresses as normal. Dox can then be removed in order to induce expression of the *Tre2-Hand1* transgene and thus continue expressing *Hand1* at high levels, rather than declining, to see if extended *Hand1* expression affects subsequent cardiomyocyte differentiation, whether differentiation is at all hindered. Likewise, ES cells can be cultured in the presence of dox to prevent *Hand1* expression prior to differentiation, and dox could be removed once differentiation is induced and subsequent cardiogenesis can be assessed. This might determine whether there is a different effect on cardiomyocytes if *Hand1* is expressed early in differentiation but not at the pluripotent ES cell stage (as in the precociously expressing D12 line).

It would also be interesting to see if under or over-expression of *Hand1* has any effect on the cellular responses to differentiation inducing signals. Various growth factors and

signalling molecules have been demonstrated to induce cardiogenesis in ES cells *in vitro* such as Wnts, TGFβ, BMPs, RA and also chemicals such as DMSO. The *Hand1*-null and *Hand1* over-expressing ES cells could be treated with some such factors to observe whether Hand1 can modulate cardiogenic inducing signals, and perhaps gain an understanding of how these signalling networks interact.

The 'gatekeeper' hypothesis that Hand1 may direct mesodermal differentiation down alternative pathways to that of the proposed 'default' cardiomyocyte fate (see section 6.1.1) has only been preliminarily explored thus far, producing interesting but inconclusive results. It is important to discover which cell lineages, if any besides cardiomyocytes, are affected by Hand1, and consequently to attempt to determine the downstream effectors of Hand1 and how they exert the commitment to particular cell lineages. It is also important to uncover a more precise mechanism for Hand1 in cardiomyocyte differentiation; in particular if there is any other effect of *Hand1* over-expression that brings about the apparent block in differentiation into the cardiac lineage other than by promoting continued proliferation of cardiomyocyte precursors. Understanding lineage commitment is particularly useful for manipulating ES cells as a tool for cell-based therapies of disease, including cardiovascular disease. However, this is limited by the inability to induce sufficient numbers of cardiomyocytes *in vitro*. There is a need to identify the instructive signals to induce cardiomyocyte differentiation and the proliferative signals to expand the population, but it is also important to maintain the correct physiological properties of the cardiomyocytes. Therefore, there is much still that needs to be understood about the role of Hand1 in commitment to the cardiomyocyte lineage.

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Appendix 1. Reagents

All reagents were of AnalaR grade and obtained from Sigma Aldrich or BDH unless otherwise stated. All solutions were made using Milli-Q purified water and autoclaved where appropriate

Southern blotting/DNA extraction

TNE buffer
50 mM Tris-HCl pH8.0; 150 mM NaCl; 100 mM EDTA pH8.0

TE pH8.0
10 mM Tris-HCl pH8.0; 1 mM EDTA pH8.0

Acid solution (Depurination solution)
250 mM HCl

Denaturation solution
1.5 M NaCl; 0.5 M NaOH

Neutralisation solution
1.5 M NaCl; 0.5 M Tris-HCl pH 7.5

20x SSC
3x NaCl; 0.3 M NaCitrate pH7.0

Hybridisation solution
50% Formamide; 5x Denhardts solution; 5x SSC; 50 mM NaP pH 6.5; 0.1% SDS;
75 μg/ml salmon sperm DNA (pre-boiled); made up to required volume with water

Low stringency wash solution
1x SSC; 0.1% SDS

High stringency wash solution
0.1x SSC; 0.1% SDS

10x Random Prime Labelling Buffer (RPLB)
100 mM Tris-HCl pH8.0; 100 mM MgCl2; 50 mM DTT; 0.5 mM dATP; 0.5 mM dGTP; 0.5 mM dTTP

Lysis buffer (tail tips)

100 mM Tris-HCl pH 8.5; 200 mM NaCl; 5 mM EDTA pH 8.0; 0.2% SDS; 100 μg/ml proteinase K added fresh

mRNA extraction

All reagents provided with Micro-FastTrack™ 2.0 Kit (Invitrogen)

Stock Buffer

200 mM NaCl; 200 mM Tris-HCl pH 7.5; 1.5 mM $MgCl_2$; 2 % SDS

Micro-FastTrack™ 2.0 Lysis Buffer

1 ml Stock Buffer + 20 μl Protein/RNase degrader (proprietary mix of proteases)

Binding Buffer

500 mM NaCl; 10 mM Tris-HCl pH 7.5 in DEPC-treated dH_2O

Low Salt Wash Buffer

250 mM NaCl; 10 mM Tris-HCl pH 7.5 in DEPC-treated dH_2O

Elution Buffer

10 mM Tris-HCl pH 7.5 in DEPC-treated dH_2O

Northern blotting

All reagents provided with NorthernMax™ Formaldehyde based system kit (Ambion)

Low stringency wash solution

1x SSC; 0.1% SDS

High stringency wash solution

0.1x SSC; 0.1% SDS

Protein extraction

RIPA buffer

50 mM Tris-HCl (pH7.6); 150 mM NaCl; 1% NP-40; 0.5% DOC; 0.1% SDS

Added fresh 0.01 mM PMSF (in isopropanol); 1x protease inhibitors; 1 mM DTT

SDS-PAGE

Component volume per 10 ml gel (ml)

Resolving gel	12%	10%	7.5%
30% acrylamide *	4	3.33	2.5
20% SDS	50 µl	50 µl	50 µl
1 M Tris pH8.8	3.75	3.75	3.75
dH2O	2.15	2.82	3.65
Temed	3.3 µl	3.3 µl	3.3 µl
20% Amps	50 µl	50 µl	50 µl

Component volume per 5 ml gel (ml)

Stacking gel	5%	8%
30% acrylamide *	0.83	1.33
20% SDS	25 µl	25 µl
1 M Tris pH6.8	0.625	0.625
dH2O	3.5	3
Temed	5 µl	5 µl
20% Amps	25 µl	25 µl

*National Diagnostics 30% protoGel solution

2x Laemlli buffer

250 mM Tris-HCl pH6.8; 4% SDS; 25% gycerol; 0.1% bromophenol blue; 5% β-Mercaptoethanol (added fresh)

1x Running buffer (1xTGS)

25 mM Tris; 192 mM Glycine; 0.1% SDS

Western blotting buffers

Transfer buffer

25 mM Tris; 192 mM Glycine; 20% methanol

10x TBS

250 mM Tris-HCl pH8.0; mM KCl; mM NaCl

TBST

0.05% Tween 20 in 1x TBS

Blocking buffer
5% non-fat milk in TBS (0.05%T)

Wash buffer
0.5% Tween 20 in 1x TBS

Stripping buffer
70 mM Tris-HCl pH6.8; 2% SDS; 100 mM β-Mercaptoethanol

Antibodies
See Appendix 4

β-Gal Assay

2x β-Gal Assay Buffer
200 mM NaPhosphate; 2 mM $MgCl_2$; 100 mM β-Mercaptoethanol; 1.5 mg/ml o-Nitrophenyl-β-D-galactopyranoside

In situ hybridisation

Whole mount *in situ* hybridisation

Hybridisation buffer
50% formamide; 1.3x SSC (pH 5.3 with citric acid); 5 mM EDTA pH 8.0; 50 µg/ml yeast RNA; 0.002% Tween-20; 0.005% CHAPS; 100 mg/ml heparin in DEPC-dH_2O

TBST
See western blotting buffers; DEPC-treated

NTMT
100 mM Tris-HCl pH 9.5; 100 mM NaCl; 50 mM $MgCl_2$; 0.05% Tween-20
Made fresh prior to use

In situ hybridisation on embryonic sections

Proteinase K stock solution
10 mg/ml proteinase K in 50 mM Tris-HCl pH 8; 5 mM EDTA; batches stored at –20°C

Proteinase K working solution
Dilute stock solution (above) 1:500 (20 µg/ml proteinase K)

Hybridisation buffer

50% formamide; 5x SSC; 1% Blocking Reagent (Roche); add 295 µl DEPC-dH_2O per 5 ml buffer (total) and heat to 65°C to dissolve blocking reagent, cool to room temperature, then add 5 mM EDTA; 0.1% Tween-20; 0.1% CHAPS; 0.1 mg/ml heparin; 1 mg/ml yeast tRNA; store batches at –20°C

Prehybridisation buffer

95% hybridisation buffer; 5% dH_2O

20x SSC

See Southern solutions; pH 4.5 with citric acid

PBST

0.1% Tween-20 in 1x PBS

B-Block

2% Blocking Reagent (Roche); 10% sheep serum in PBST

Dissolve Blocking Reagent in PBS at 95°C and cool before adding sheep serum and Tween-20, store batches at –20°C.

NTMT

See whole mount *in situ* hybridisation buffers

Riboprobes

See Appendix 5

Cardiomyocyte isolation

Low Calcium medium

120 mM NaCl; 5.4 mM KCl; 5 mM $MgSO_4$; 5 mM sodium pyruvate; 20 mM Glucose; 20 mM Taurine; 10 mM Hepes/NaOH pH6.9 in dH_2O, made fresh and filter sterilised.

KB medium

85 mM KCl; 30 mM K_2HPO_4; 5 mM $MgSO_4$; 1 mM EGTA; 5 mM sodium pyruvate; 5 mM Creatine; 20 mM Taurine; 20 mM Glucose; 2 mM Na_2ATP in dH_20 made fresh and filter sterilised.

Enzyme medium

Low Ca^{2+} Medium supplemented with 1 mg/ml collagenase B (Roche) and 30 µM $CaCl_2$

Appendix 2. Primer sequences

Number	Sequence	Region	Source (where applicable)
136 (PrD)	5'-CCACTAGGATCGCACGTGCA-3'	Hand1 genotyping (S)	(Riley et al., 1998)
172 (PrA)	5'-GCGCCTGGCTACCAGTTACA-3'	Tet-Off-Hand1 genotyping (S)	(Riley et al., 2000)
173 (PrB)	5'-AGAAGGGCCCAGGGAAGACT-3'	Tet-Off-Hand1 genotyping (AS)	(Riley et al., 2000)
294	5'-GTGGGCCGCTCTAGGCACCAA-3'	β-Actin (S)	(Lyons et al., 1995)
295	5'-CTCTTTGATGTCACGCACGATTTC-3'	β-Actin (AS)	(Lyons et al., 1995)
340	5'-CGGAATTCAAACGAAAAGGCTCAGGACC-3'	bHLH (S)	
341	5'-CCGCTCGAGCAAGTAGGCGATGTAACTG-3'	bHLH (AS)	
351 (PrE)	5'-TCAGCAACGAATGGGAACGC-3'	Hand1 genotyping (AS)	(Riley et al., 1998)
353 (PrF)	5'-GCAAAGCTGCTATTGGCCGC-3'	Neo^r Hand1 genotyping (S)	(Riley et al., 1998)
357	5'-CAGCGCATTAGAGCTGCTTA-3'	Tet-Off transactivator (S)	
358	5'-TACCCACCGTACTCGTCAAT-3'	Tet-Off transactivator (AS)	
392	5'-CCGGAATTCTGATGAACCTCGTGGG-3'	Hand1 coding for EGFP fusion (S) (R1)	
393	5'-CGGGGTACCGTCTGGTTTAGCTCCA-3'	Hand1 coding for EGFP fusion (AS) (Kpn)	
403 (PrC)	5'-GGGGTGGGGTGGGATTAGAT-3'	Neo^r Tet-Off-Hand1 genotyping (S)	
426	5'-ATGGTGAGCAAGGGCGAGGA-3'	EGFP in 12.5 (S)	
427	5'-CCTCCTTGAAGTCGATGCCC-3'	EGFP in 12.5 (AS)	
512	5'-AAGCTTTCGGGCTGCTGAGG-3'	Hand1 across intron (AS)	
513	5'-CCCCTGGAGCTCTATAGGGA-3'	GAPDH (S)	
514	5'-CCTTCTCGTTCACTCCCATG-3'	GAPDH (AS)	
575	5'-ATCTCAATGGCTAAGGCGTC-3'	Tet-Off transactivator (AS – short product)	
622	5'-GGCTGGAGTGCGATCTT-3'	Tet-LacZ genotyping (S)	T. Mohun
623	5'-TGACGCGATCGGCATAA-3'	Tet-LacZ genotyping (AS)	T. Mohun
624	5'-CTGGGCGCTGGAGCTAAACCAG-3'	Tet-Hand1 genotyping (S)	T. Mohun
625	5'-CAGTAGCCTCATCATCACTAGAT-3'	Tet-Hand1 genotyping (AS)	T. Mohun
675 (PrG)	5'-GATGGGACTGGAGAAGACCA-3'	Tre2-Hand1 transgene specific (S)	PRIMER3 software (SDSC Biology

			Workbench 3.2)
676 (PrH)	5'-GAAGTCAGATGCTCAAGGGG-3'	Tre2-Hand1 transgene specific (AS)	PRIMER3 software
694	5'-TCACTGTGCCTGAACTTACC-3'	Tubulin (S)	(Dell'Era et al., 2003)
695	5'-GGAACATAGCCGTAAACTGC-3'	Tubulin (AS)	(Dell'Era et al., 2003)

Appendix 3. Primer sequences for quantitative real-time PCR

Region	**Sequence**	**Source/designed using**
α-cardiac actin (S)	5'-GCCCCTAGCACGCCTACA-3'	Primer Express software (version 2.0, Applied Biosystems)
α-cardiac actin (AS)	5'-GGTCTCCTCGTCGTCACACA-3'	Primer Express software
Brachyury (T) (S)	5'-ACCCCAATGCCATGTACTCTT-3'	Primer Express software
Brachyury (T) (AS)	5'-TCCCCGTTCACATATTTCCA-3'	Primer Express software
ANF (S)	5'-TTCCTCGTCTTGGCCTTTTG-3'	(Fijnvandraat et al., 2003b)
ANF (AS)	5'-CCTCATCTTCTACCGGCATCTTC-3'	(Fijnvandraat et al., 2003b)
Chisel (S)	5'-ATCCAGAGAGCAGGGCTAAGAC-3'	Primer Express software
Chisel (AS)	5'-CCTGGATGGCTCTGACGTT-3'	Primer Express software
Cx40 (S)	5'-GGAAGACGGGCTGTTCCA-3'	(Fijnvandraat et al., 2003b)
Cx40 (AS)	5'-CCCATTTCAGAAAACAAACACA-3'	(Fijnvandraat et al., 2003b)
Cx43 (S)	5'-CTCCTGCCGCAATTACAACA-3'	Primer Express software
Cx43 (AS)	5'-GCCTGCCCCATTCGATT-3'	Primer Express software
Eomes (S)	5'-CACCATCAGATTCCACAGGTTT-3'	Primer Express software
Eomes (AS)	5'-GAAGTGGAAGAAGATGCTGCAT-3'	Primer Express software
Fgfr1 (S)	5'-GCCAAGACGGTGAAGTTCAA-3'	Primer Express software
Fgfr1 (AS)	5'-ATTTTTCAACCAGCGCAGAGT-3'	Primer Express software
GAPDH (S)	5'-TGTCAGCAATGCATCCTGCA-3'	(Lekanne Deprez et al., 2002)
GAPDH (AS)	5'-CCGTTCAGCTCTGGGATGAC-3'	(Lekanne Deprez et al., 2002)
GATA1 (S)	5'-AGCTTCAACATAAACGGACTCAAC-3'	Primer Express software
GATA1 (AS)	5'-GGTCGGCCAAAACCACTTC-3'	Primer Express software
GATA4 (S)	5'-TGAAGAGATGCGCCCCATCAA-3'	(Fijnvandraat et al., 2003b)
GATA4 (AS)	5'-ATAGCCTTGTGGGGACAGCT-3'	(Fijnvandraat et al., 2003b)
Hand1 (S)	5'-TGAGTGCATCCCCAATGTG-3'	Primer Express software
Hand1 (AS)	5'-GCCAGCACGTCCATCAAGTA-3'	Primer Express software

Hand2 (S)	5'-TCGCCTACCTCATGGATCTG-3'	Primer Express software
Hand2 (AS)	5'-TCTTTCACGTCGGTCTTCTTGA-3'	Primer Express software
Irx4 (S)	5'-ACGGTGCCCCAAGACGT-3'	(Fijnvandraat et al., 2003b)
Irx4 (AS)	5'-TGGAAACCCATTTAAAGCAGTTC-3'	(Fijnvandraat et al., 2003b)
Mef2c (S)	5'-CCCCTTCGAGATACCCACAA-3'	(Fijnvandraat et al., 2003b)
Mef2c (AS)	5'- GAAGGTCTGGTGAGTCCAATGG-3'	(Fijnvandraat et al., 2003b)
Mlc2v (S)	5'-GGGAGATGCTGACCACACAAG-3'	(Fijnvandraat et al., 2003a)
Mlc2v (AS)	5'-TTCAGGGCTCAGTCCTTCTCTT-3'	(Fijnvandraat et al., 2003a)
Nkx2.5 (S)	5'-CTATGCCCTGTCCCTCGGAT-3'	(Fijnvandraat et al., 2003b)
Nkx2.5 (AS)	5'-CTCCCGGTCCTAGTGTGGAA-3'	(Fijnvandraat et al., 2003b)
SRF (S)	5'-ACCAGGTGTCGGAATCTGACA-3'	Primer Express software
SRF (AS)	5'-TGTCTGGATTGTGGAGGTGGTA-3'	Primer Express software

Appendix 4: Antibody information

Primary antibody	Dilution	Secondary antibody	Dilution	Application
Hand1-C terminus (Santa Cruz)	1:500	Goat-HRP (Santa Cruz)	1:5000	Western
EGFP (Clontech)	1:1000	Mouse-HRP (Amersham)	1:5000	Western
Cleaved Caspase 3 (Cell Signalling Technology)	1:100	Rabbit-FITC (Santa Cruz)	1:100	IF
Phospho-histone H3 (Upstate)	1:200	Rabbit-TRITC (Dako)	1:50	IF
CDK4 (Santa Cruz)	1:500	Rabbit-HRP (Amersham)	1:5000	Western
Cyclin D2 (AbCam)	1:500	(Mouse-HRP) (Amersham)	1:5000	Western
GAPDH (Chemicon)	1:2000	Mouse-HRP (Amersham)	1:5000	Western
MF20 (Developmental Studies Hybridoma Bank)	1:10	Mouse-TRITC (Dako)	1:30	IF
Nkx2.5 (Santa Cruz)	1:100	Goat-FITC (Dako)	1:20	IF

IF, Immunofluoresence

Appendix 5: *In situ* hybridisation riboprobes

Riboprobe (AS)	Source/reference	Linearise with	RNA Polymerase
ANF	Kuo et al., (1997).	*Hind*III	T7
Hand1	Cserjesi et al., (1995)	*Not*I	T7
Mlc2v	Kubalak et al., (1994)	*Kpn*I	T7
Nkx2.5	Lints et al., (1993)	*Xba*I	T7
tTA	In house	*Hind*III	T7
tTA (S)	In house	*Not*I	T3
Wnt11	Cai et al., (2003)	*Hind*III	T3